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MICROBIAL SPECIATION AND BIOFOULING POTENTIAL OF COOLING WATER USED BY ONTARIO HYDRO

Report No 84-276-K

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ABSTRACT

The cooling water composition and microbial components of biofilms attached to stainless steel wafers submerged in three lake water types were evaluated to determine whether their biofouling potential differed in a predictable manner. The composition of the lake waters was different which affected biofilm composition, where the predominance of specific microbial groups varied between test systems and with time. Some prediction of biofouling potential was possible, and it was concluded that the cooling water in the vicinity of Bruce NGS had the lowest biofouling potential whereas greater biofouling could be expected in the Pickering and Nanticoke stations.

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EXECUTIVE SUMMARY

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Biofouling is the term used to describe the attachment and growth of aquatic micro-organisms on submerged surfaces of industrial importance. Biological analyses of deposits taken from failed heat exchangers at Ontario Hydro generating stations have indicated the involvement of biofouling in these failures. More information on the problem is required before mitigative procedures can be devised. The objectives of this investigation were to demonstrate that microbes preferentially attach to metal surfaces, to ascertain whether distinct microbial species play different roles in the process of biofilm attachment and development and to determine that if microbial composition of the major cooling waters used by Ontario Hydro differs, whether this allows prediction of their biofouling potential.

Closed loop cooling water systems, containing service water from either Bruce, Pickering or Nanticoke stations in which were submerged stainless steel wafers, were set up. Microbiological analyses were performed on the water and attached biomass over a 131 day period. Visual observation of the biofilm in situ was carried out using a scanning electron microscope.

It was demonstrated that microbes do preferentially adsorb to metal surfaces, such that the numbers in the biofilm were from 3 to 400 times greater than those found in the water. Observation with a scanning electron microscope showed that attachment did not occur in even layers but built up in patches. Examination

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of microbial speciation in the biofouling process revealed that biofilm composition changed as it developed and was different in the three test systems. Filamentous organisms were responsible for early colonization of the wafers in the Bruce bioreactor, while aerobic heterotrophs predominated in the Nanticoke and Pickering systems. The aerobic heterotrophs, a highly diverse and nutritionally versatile group, dominated biofilm composition at 61 days, after which time their importance declined as numbers of pseudomonads and ammonifiers increased. Filamentous morphology did not necessarily enhance adsorption, as nutrient status of the cooling water and numbers of microbes were equally important. Clear differences in water composition between the three sources were demonstrated, although these differences were not seen consistently in the developing biofilm. Prediction of biofouling based on different species was limited, but analysis of total numbers of microbes allowed some assessment. On this basis it was concluded that cooling water in the vicinity of Bruce NGS had the lowest biofouling potential and that greater biofouling could be expected in the Pickering and Nanticoke stations.

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To Mr. F.J. Kee
Director of Research

MICROBIAL SPECIATION AND BIOFOULING POTENTIAL OF COOLING WATER USED BY ONTARIO HYDRO

1.0 INTRODUCTION

Biofouling is the term used to describe the attachment and growth of aquatic micro-organisms on submerged surfaces of industrial importance. Biological colonization of surfaces such as heat exchanger tubes is thought to precede entrainment of inorganic particles, with the resultant adherent layer of material having a detrimental affect upon heat exchanger performance (Corpe and Winters, 1980). Problems frequently associated with biofouling, increased operating and maintenance costs at generating stations, corrosion and unscheduled power outages have been documented elsewhere (Ontario Hydro, 1982; Battaglia et al, 1981; Garey et al, 1980; Somerscales and Knudsen, 1981; Lavi and Veziroglu, 1978). An increased awareness of such problems at Ontario Hydro has led to biological analysis of deposits taken from heat exchangers that failed due to unacceptable reduction in cooling capacity or tube wall perforation. Failure of Incoloy-800 tubes in a moderator heat exchanger (HX) at Pickering NGS 'B' (Ontario Hydro, 1983 b), tubes of the same alloy in a moderator HX at Bruce NGS 'A' (Ontario Hydro, 1983 c) and of pure copper tubes in a turbine lube oil HX also at Bruce NGS 'A' (Ontario Hydro, 1984) were attributed, in part, to biofouling. The role of micro-organisms in HX failure could not be fully determined because the analyses were 'after the fact'.

It has become apparent that more information on biofouling at Ontario Hydro generating stations is required prior to the development of mitigation procedures. However, biofouling has been cited as a difficult problem to control because of its variable nature and extreme site specificity (Jorden, 1980). This site specificity has been demonstrated by the performance of Incoloy-800 moderator HX tubes that have had many problems at Pickering NGS but fewer at Bruce NGS. There are some differences in design and length of service between HXs at the two stations, but it is likely that the different sources of cooling water have also been important.

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The aims of this investigation were to:

i) unequivocally demonstrate that viable micro-organisms will attach to and grow on submerged metal surfaces,

ii) determine whether distinct microbial groups play different roles in the process of biofilm attachment and development,

iii) ascertain if microbial content of cooling waters differs and whether this affects the biofouling potential of specific lake water sources, thereby allowing categorization of Ontario Hydro generating stations in order of susceptibility to biofouling.

Historically, within the power industry there has been some scepticism that microbes attach to metal surfaces, or that there is sufficient attachment for a biofouling layer to develop, so the first objective addresses these concerns. The involvement of specific microbial groups in biofilm development has received little attention. The qualitative analyses that do exist are highly selective as nuisance organisms within the biofilm were studied rather than evaluation of the role of general microbial populations to be found in aquatic systems. Therefore, a more realistic approach to assess biofouling, that may also indicate mechanisms of biofilm development, is required. In fact, it has been stated that study of cooling waters as well as direct examination of metal surface to provide information on relative numbers and kinds of microbial cells present is necessary, before completely satisfactory control of the problem is possible (Corpe and Winters, 1980). The third objective is oriented towards the site specific aspect of biofouling. This study will provide information on cooling waters used by Ontario Hydro and may shed some light on the variable performance of HXs at different generating stations.

The approach taken in this investigation was to set up three bioreactors in which stainless steel wafers were exposed to water from either Lakes Ontario, Huron or Erie. The microbial populations in the water phase and at the metal-water interface were monitored using several techniques. In the report the data and discussion is presented in sections covering biofilm development, microbial speciation and biofouling potential.

2.0 METHODS AND MATERIALS

2.1 Bioreactor Design

Each bioreactor consisted of an autoclavable Nalgene vessel (4 L capacity) sealed with a lid through which ports had been

drilled. Through these ports were placed rigid plastic rods which held stainless steel wafers (304 L) that were used to mimic heat exchanger tube surfaces. Water surfaces were cleaned in a protein scavenging solution (Contrad-70) and degreased in 95% ethanol. The wafers were of two sizes, one (12.5 cm^2) for viable count work and the other (3 cm^2) for scanning electron microscopy. The ports were closed with rubber seals. The lake water in each bioreactor was stirred continuously throughout the experiment using a magnetic stirrer to provide an annular flow system. The wafers were placed approximately parallel to the direction of water flow. To keep the system aerated, compressed air was passed through water and glass wool traps to remove particulates and dried by passage through anhydrous calcium sulphate prior to delivery to the bioreactors. All the equipment used to construct the bioreactor was sterilized by either alcohol (95%) wash or autoclaving (20 min at 115°C). Periodically the temperature and the pH (Radiometer PHM82 - combined glass electrode) of the lake waters were recorded so that should adverse conditions occur that may affect microbial viability then they would have been detected.

There were three bioreactors containing water from either Lake Ontario, or Lake Huron or Lake Erie. The water was collected within two days from the start of the experiment from the intake areas of Pickering, Bruce and Nanticoke generating stations. Throughout this report the lake water types will be referred to by the name of the station from which they were taken. Unused lake water was stored at 4°C and used to replenish volumes lost during sampling. In this way additions of fresh lake water minimized nutrient limitation and, as the volumes were added immediately after sampling, there was sufficient time for microbial populations to stabilize before another sample point. Microbial populations were enumerated in the water phase at the start of the experiment and in the water phase and metal-water interface after 27, 61 and 131 days.

2.2 Microbiological Analysis

Water samples were taken directly from the bioreactors and a series of tenfold dilutions prepared using sterile phosphate buffer (0.25 M, pH 7.2). Biofilm samples were taken by scraping the attached biomass from the wafer, using an alcohol cleaned plastic knife, into a pre-weighed weighing boat and the weight recorded on an analytical balance. Using a known volume of buffer the wafer and knife were rinsed to ensure that all the material had been collected and this constituted the first dilution. After extensive vortex mixing to disburse the biomass, additional dilutions were performed.

It should be noted that the microbial numbers obtained for attached biomass were underestimated because of two factors. The biomass sampled was bulky and very hydrated thereby contributing an unknown amount of dilution to the first dilution level. Secondly, the aggregates of biomass could not be completely homogenized and so the assumption held for the plate count procedure, that one colony arises from one microbial cell, probably did not hold true.

The microbial groups enumerated were actinomycetes, aerobic heterotrophic bacteria, ammonifying bacteria, anaerobic sulphate-reducing bacteria, fungi and members of the Pseudomonadaceae family. Details of methods and materials for the isolation and quantification of aerobic heterotrophs, ammonifying bacteria, anaerobic sulphate reducers and fungi can be found elsewhere (Ontario Hydro, 1983 a).

The actinomycetes were cultured on a starch-casein medium which had been modified with an antifungal agent, cycloheximide, to enhance its selectivity (Appendix A). Inoculated plates were incubated at 28°C and assessed for growth at 5 and 10 days. Counting of adherent fibrous colonies was performed with the aid of a stereo-microscope.

The Pseudomonadaceae family encompasses a wide range of organisms all of which are unlikely to be isolated on any one medium. Flo Agar (BBL) (Appendix A), was used to identify pseudomonads as this culture medium encourages the production of fluorescein which is commonly secreted by pseudomonads. Spread plates of the inoculated medium were inverted and incubated at 25°C for 48 h prior to counting.

The majority of microbial groups were enumerated using a spread plate (lawn) method with three replicates at each dilution for each microbial type. The results were expressed as numbers of cells per 1 cm^3 for both water and biofilm values with the assumption that one cell gave rise to one colony. The ammonifying bacteria and anaerobic sulphate reducers were quantified using five replicates per dilution as part of a Most Probable Number (MPN) method, with the results also expressed as cells per 1 cm^3. Limited space in the bioreactors did not allow for replication of the wafer sampled at all the time points. However, there was one set of replicates measured in order to obtain an estimate of wafer to wafer variability.

2.3 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to provide additional information on the process of biofilm attachment and development. Identification and enumeration of microbial genera

necessitates destructive methods and so valuable information on the physical appearance of biofilms is lost. As samples for observation using a scanning electron microscope are merely dried, and in some cases shadowed, this instrument lends itself particularly well to the examination of in situ biofilms.

Wafers for SEM examination were removed from the bioreactors and rinsed in sterile, filtered (cellulose nitrate membrane filter 0.2 µm pore size) 0.25 M phosphate buffer to remove any material which was not properly attached. The rinsed wafers were immediately placed in a 4% glutaraldehyde solution (in aqueous phosphate buffer) to fix the material and left for one hour. At which time the wafers and attached material were rinsed in filtered phosphate buffer, and then progressively dehydrated by immersion for 5 min per stage in aqueous acetone solutions of 5%, 10%, 20%, 40%, 80%, 90%, 95% and 100% acetone, with one repeat exposure in fresh solution at the initial (5%) and final (100%) stages.

The specimens were sputter coated with gold-palladium (Au-Pd) to improve visualization of the attached material and then scanned at an accelerating voltage of 20 kV using a JEOL JS14-35C scanning electron microscope. Some samples were shadowed with carbon rather than Au-Pd to remove interference when the SEM was used in combination with an energy dispersive x-ray analysis (EDAX) system (SW 9100/60). EDAX was used to provide a semi-quantitative measurement of elements present in the material attached to the stainless steel wafers. Further information on the EDXA technique can be obtained in a report by Sandloehken (1983).

3.0 RESULTS AND DISCUSSION

3.1 Biofilm Development

Visual observation of the developing biofilm, using a scanning electron microscope, revealed an uneven distribution of attached biomass within 27 days, which increased in density but maintained this appearance throughout the test (see Appendix B). It has been cited that bacterial adhesion occurs rapidly, forming continuous monolayers, possibly several cells thick, 10 to 20 minutes after surface-water contact (Characklis, 1973). Also glass coverslips exposed to saline water became covered in a biofilm of filamentous bacteria, which were evenly distributed and surrounded by entrapped debris of organic origin (Pedersen, 1982). However, there is reported work that concurs with the findings in this study. Scanning electron micrographs of microbial attachment to aluminum pipe revealed widely separated patches of filamentous and rod shaped organisms after 18 days incubation, which become less defined after 32 and 45 days (Little and Lavoie, 1980). This lack of a confluent biofilm

layer indicates that microbial adhesion is largely controlled by surface energies. It is thought that materials with critical surface tensions similar to those of bacterial cells (20-30 dynes/cm) exhibit minimal biological adhesion, whereas materials with critical surface tensions below or above this value, such as metals, support bioadhesion to a greater extent (Baier, 1980). Surface topography may also affect attached biomass distribution as it has been demonstrated that surface roughness enhanced microbial adhesion to polystyrene, although the adherent cells were not particularly concentrated in the grooves (Baker, 1984).

The presence of an unevenly distributed layer of biological material has an important implication for biological corrosion processes. Microbes are thought to contribute to corrosion by creating differential oxygen concentration cells (Iverson, 1968). Underneath attached biomass there will be oxygen depletion due to its utilization in respiration processes, hence creating uneven distribution in relation to levels on adjacent areas of bare metal. This mechanism would appear to be feasible, judging by the pattern of attached biomass observed on the stainless steel surfaces.

Monitoring of attached biofilms using energy dispersive x-ray analysis is limited to elements with atomic weights of 11 or greater, which excludes the primary biological elements of carbon, oxygen, nitrogen and hydrogen. Furthermore, electron beam penetration to a depth of 3 μ giving a high background count of metallic elements and low levels of other important biological elements allows for only qualitative analysis. Values for phosphorous, sulphur, sodium and potassium were highly variable, but the rate at which counts for elements accumulated was very slow, indicating a lot of biological material (atomic weight <8) was present. Refinement of this technique could lead to ready identification of biological material performed simultaneously with metallurgical analyses.

Prior to examination of the results from the bioreactors it was determined that conditions had remained conducive to microbial growth. The pH of the lake waters from initial collection to end of test covered a range of pH 8.24-8.49 for Bruce, pH 8.29-8.56 for Pickering and pH 8.26-8.46 for Nanticoke. The slight increase in alkalinity for all the water types was probably due to microbial activity, and was sufficiently small to have caused no stress. Although microbial effects on pH would not be manifested in the water of a once through cooling system, an increase in alkalinity within the biofilm is possible. This could increase phosphate precipitation on the metal surface, which may have an important consequence as it has been observed that a biofilm layer became more adherent when phosphate scaling occurred (Rippon, 1979).

The water temperature in the bioreactor ranged from 22.0-22.7°C, with no appreciable difference between them. Although there is considerable seasonal variation, the temperature in the bioreactors is similar to maximum, mean daily values recorded in August near generating stations of approximately 21°C for Lake Ontario (Ontario Hydro, 1983 d) and 20-22°C for Lake Erie (Polak, 1977).

The approach taken in this study was to monitor major aquatic microbial groups that could potentially attach to submerged metal surfaces. Information of this type is limited as previous investigations were generally orientated towards discussion of possible nuisance organisms (Creedon, 1979), or the extent of classification was minimal (Eaton et al, 1980). Numerically dominant groups within lakes have been cited as Actinomycetales, Pseudomonadaceae and Bacillaceae (Kuznetsov, 1970) and hence the first two groups were selected for study. Aerobic heterotrophic bacteria were enumerated as a means of encompassing a broad range of organisms, including the genus Bacillus. Ammonifying microbes are another important physiological group chosen for investigation. The importance of filamentous microbial forms in attachment has been mentioned by several researchers (Weiss, 1970; Corpe, 1970), and so fungi, with string-like mycelial morphology, and actinomycetes, which tend to form branched filaments, were chosen as major representatives. Anaerobic sulphate reducers were included because of their ubiquitous distribution in aquatic environments and their importance in microbial corrosion processes.

Analysis of these groups was performed on the water and attached biomass over a 131 day period and a comparison of numbers in the two phases is presented in the following figures and discussion. The results obtained for actinomycetes can be seen in Figure 1. In all cases the values for the biofilm were greater than, and almost parallel to those for the water. In the Nanticoke and Pickering bioreactors the values for both biofilm and water remained almost constant between 27 and 61 days, followed by a sharp rise in numbers from 61 to 131 days of testing. The pattern for Bruce was different, with far greater numbers attached to the wafer after 27 days of incubation followed by a much more gradual increase in biofilm numbers. The final number of actinomycetes in the biofilm was high, around 10^6-10^7 cells/cm^3 and similar for all the lake types.

Actinomycetes were more prevalent in the Bruce reactor than in Nanticoke and Pickering reactors in the early stages of biofilm development, although the greatest difference between biofilm and water was seen in the Nanticoke reactor at 61 and 131 days.

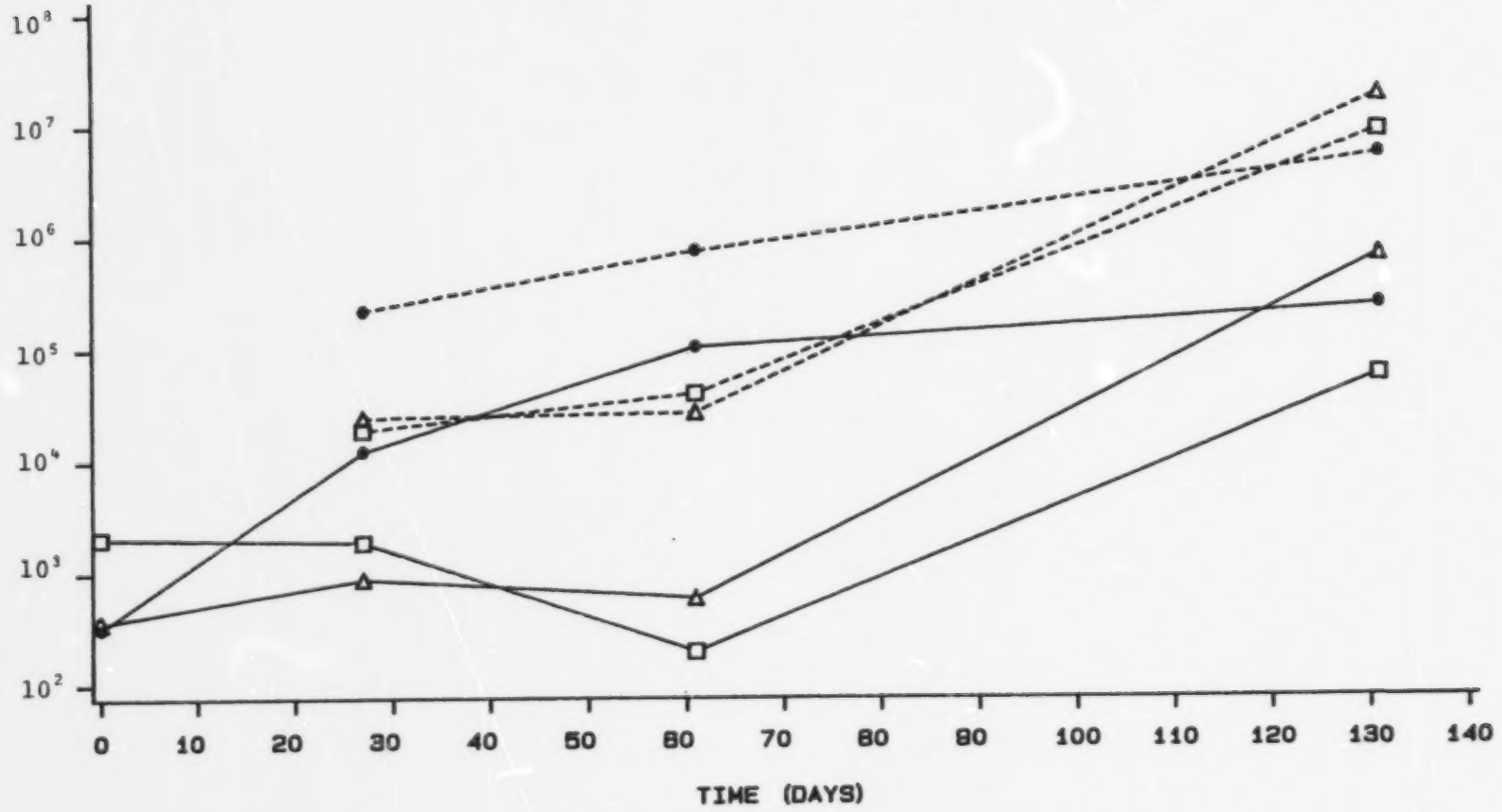

FIGURE 1. INVOLVEMENT OF ACTINOMYCETES IN BIOFILM DEVELOPMENT

BACTERIAL NUMBERS IN BIOFILM (-----) AND LAKEWATER (———) IN BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (●), NANTICOKE TGS (□), AND PICKERING NGS (△).

In order to determine whether the apparent differences seen in the figure were statistically significant a modified Students' t-test was performed and the tables can be found in Appendix C. Examination of the table shows that the number of actinomycetes in the biofilm was significantly different to, and greater than, the values obtained in the lake water for each bioreactor, at all times. Therefore naturally occurring populations of actinomycetes preferentially attach to and colonize stainless steel wafers submerged in water.

Figure 2 shows the results obtained for aerobic heterotrophic bacteria. There appears to be more bacteria in the biofilm than in the water at all times for Bruce, Nanticoke and Pickering bioreactors. This difference is confirmed by statistical analysis (Appendix C). As was the case for actinomycetes, the difference between biofilm and water values ranged from about 3 to 200 times more in the biofilm, with the greatest difference in the Nanticoke system at 61 and 131 days.

It can be seen that the rate of attachment and growth on stainless steel wafers was most rapid between 0-61 days, with a very gradual increase up to 131 days. In one of the few studies of biofilm composition, the numbers of bacterial groups were monitored in a closed loop model consisting of river water and 70:30 (Cu:Zn) brass condenser tubing. Biofilm development was measured over a 60 day period at which time bacterial growth had stabilized at a level of roughly 1×10^{10} cells/cm^2 (Tarbuck and Wyborn, 1980). In the work presented here a rapid increase in numbers of attached cells was also seen in the initial phase but this continued until day 60. After 131 days exposure the aerobic heterotroph numbers in the biofilm seemed to have stabilized but at a much lower level, ranging from 3.6×10^5 cells/cm^2 for Bruce up to 1.2×10^6 cells/cm^2 for Nanticoke. As similar methods for enumerating aerobic heterotrophs were used in the two studies, it would seem that the river, a fairly polluted cooling water source, had a greater biofouling potential for brass surfaces than the Great Lakes water had for stainless steel.

The third microbial group studied was the ammonifiers and the results can be seen in Figure 3. The pattern of change and the relationship between biofilm and water was less clear than for the two groups discussed previously. Biofilm and water numbers in the Pickering bioreactor were consistently higher than for Bruce and Nanticoke, except at 61 days where the Nanticoke water value exceeded that for Pickering. Statistical analysis revealed that biofilm values were greater than water values, except for Pickering 27 day and Nanticoke 61 day points (Appendix C). The biofilm to water ratios were generally low but increased substantially by 131 days for Pickering and Nanticoke (Appendix C).

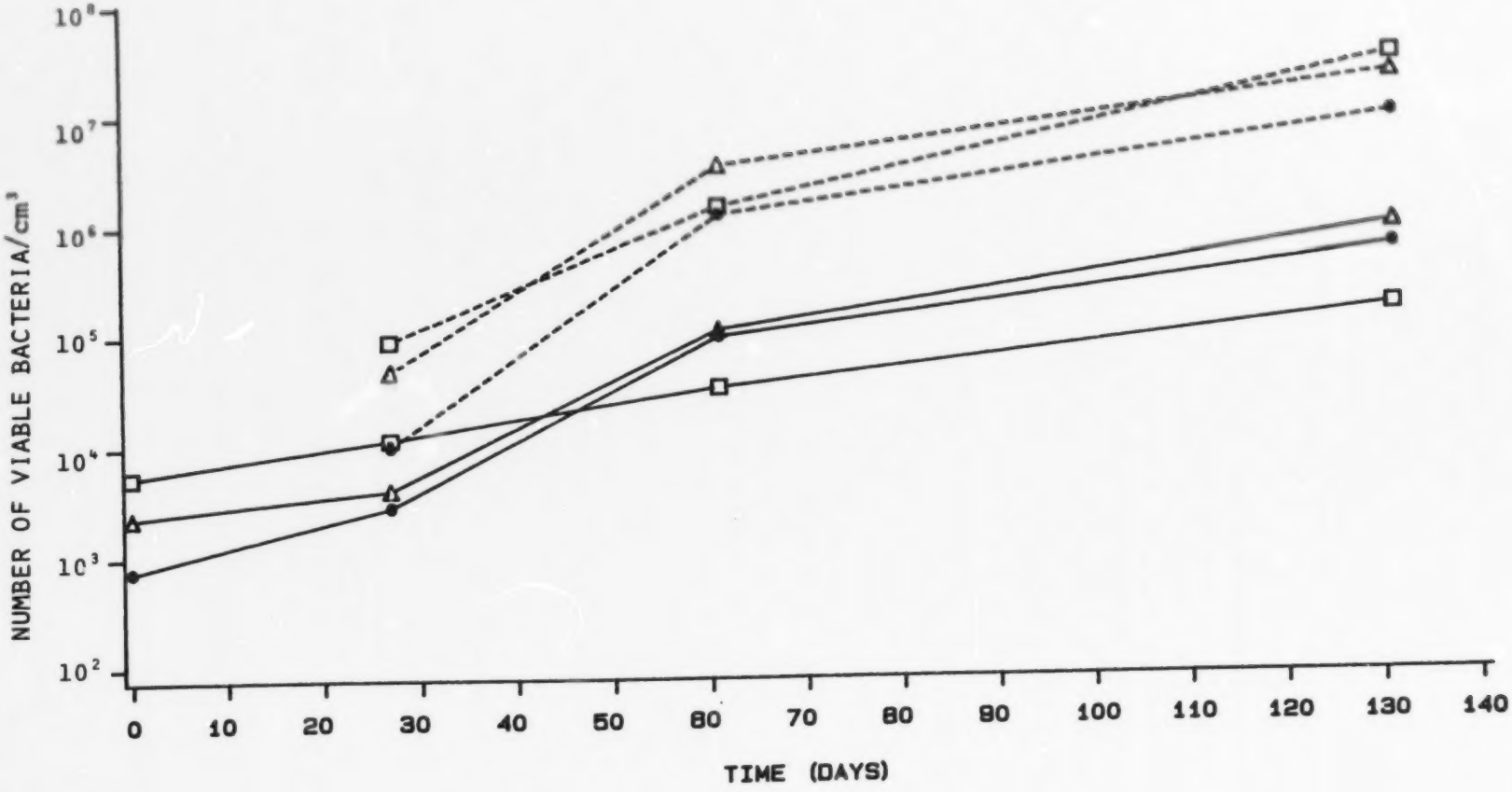

FIGURE 2. INVOLVEMENT OF AEROBIC HETEROTROPHIC BACTERIA IN BIOFILM DEVELOPMENT

BACTERIAL NUMBERS IN BIOFILM (-----) AND LAKEWATER (———) IN BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (●), NANTICOKE TGS (□), AND PICKERING NGS (△).

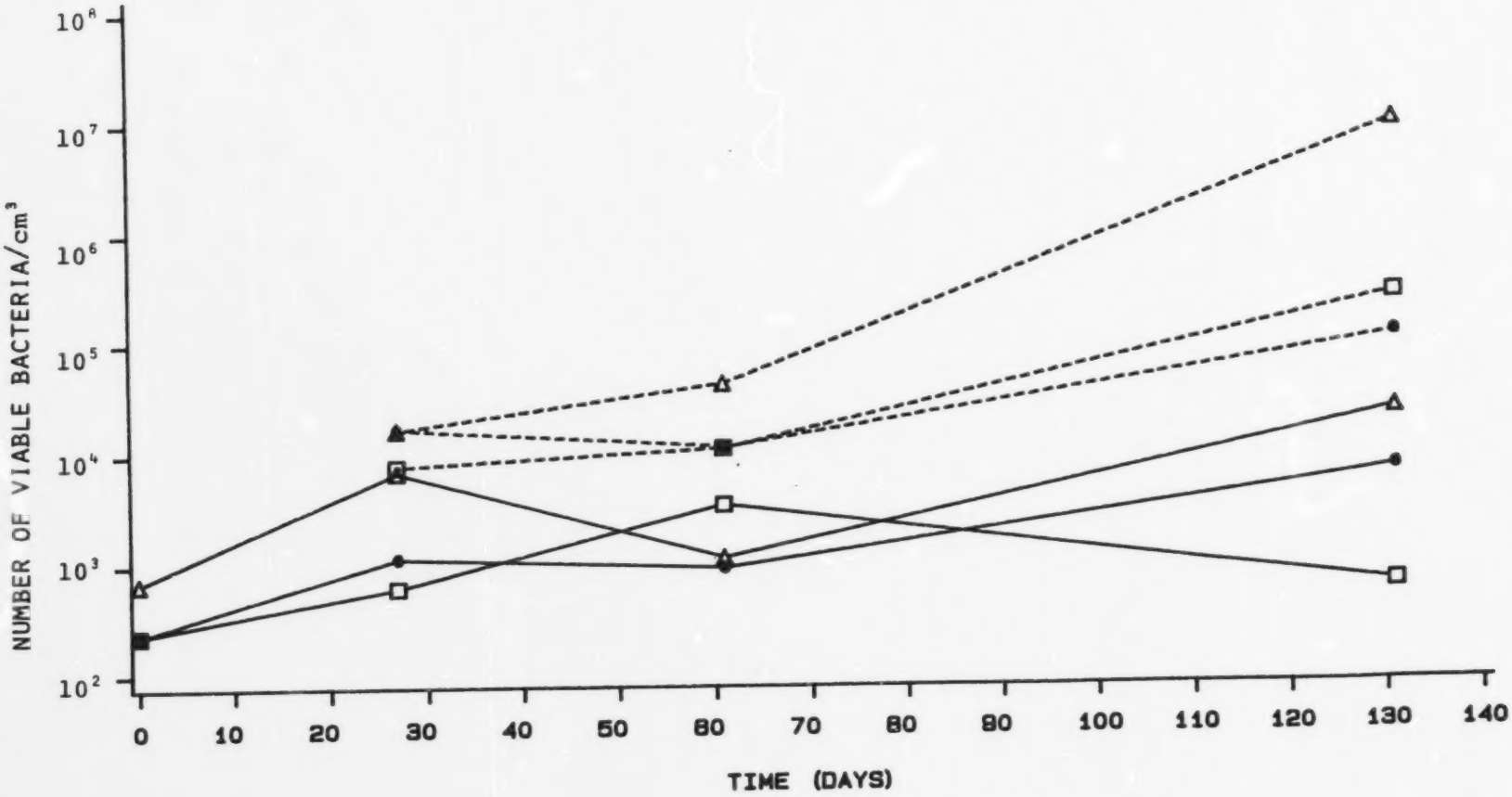

FIGURE 3. INVOLVEMENT OF AMMONIFYING BACTERIA IN BIOFILM DEVELOPMENT.

BACTERIAL NUMBERS IN BIOFILM (-----) AND LAKEWATER (———) IN BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (●), NANTICOKE TGS (□), AND PICKERING NGS (△).

The significance of ammonifiers in the developed biofilm is of interest when related to their physiology. The substrate for ammonification is nitrogenous organic matter and so it is possible that this substrate is in limited supply over the biofilm growth phase. As the microbial population increased there would be a progressively greater availability of nitrogenous organics. In addition, as biomass ages there will be a higher incidence of cell death and lysis, resulting in the release of organics, which in turn could stimulate ammonifying activity. Hence, it is possible than ammonifying micro-organisms will become an increasingly important constituent of the biofilm as it ages.

Ammonia, a product of ammonification may be involved in microbial corrosion processes as the biofilm develops. Under aerobic conditions, ammonia produced by deamination is readily assimilated by a wide range of organisms, although organic acids, which are produced simultaneously, may be corrosive. However, under anaerobic conditions ammonia will accumulate, and as brass is susceptible to attack by ammonia (Tarbuck and Wyborn, 1980), it would be particularly vulnerable if the biofilm became oxygen limited, due to either stagnant water conditions or to the development of a thick, diffusion limiting film.

Release of ammonia may also affect biofilm development. A relationship between the density of ammonifying bacteria and the concentration of ammonium salts in water has been demonstrated in deep lakes (Kuznetsov, 1970). It is possible that ammonia released from biofilms will combine with carbonates in the cooling water, with the resultant precipitate contributing to the mass of the biofilm.

The total number of ammonifiers, which ranged from 4.7×10^3 cells/cm^2 (Bruce) to 4×10^5 cells/cm^2 (Pickering), is low when compared to the plateau value of 7×10^9 cells/cm^2 obtained by Tarbuck and Wyborn (1980). The biofilm development did not reach a plateau in any of the bioreactors and so some increases in ammonifying numbers was expected. There was some concurrence between the two studies as the relative importance of ammonifying bacteria as a biofilm constituent was similar.

The extent and rate of attachment of anaerobic sulphate reducing bacteria to stainless steel wafers can be seen in Figure 4. The most rapid attachment occurred in the initial 27 days after which time there was virtually no discernable change in biofilm numbers.

The numbers of sulphate reducers present in the lake waters prior to experimentation was low (6 cells/cm^3 Bruce to 70 cells/cm^3 Pickering), relative to aerobic heterotrophs

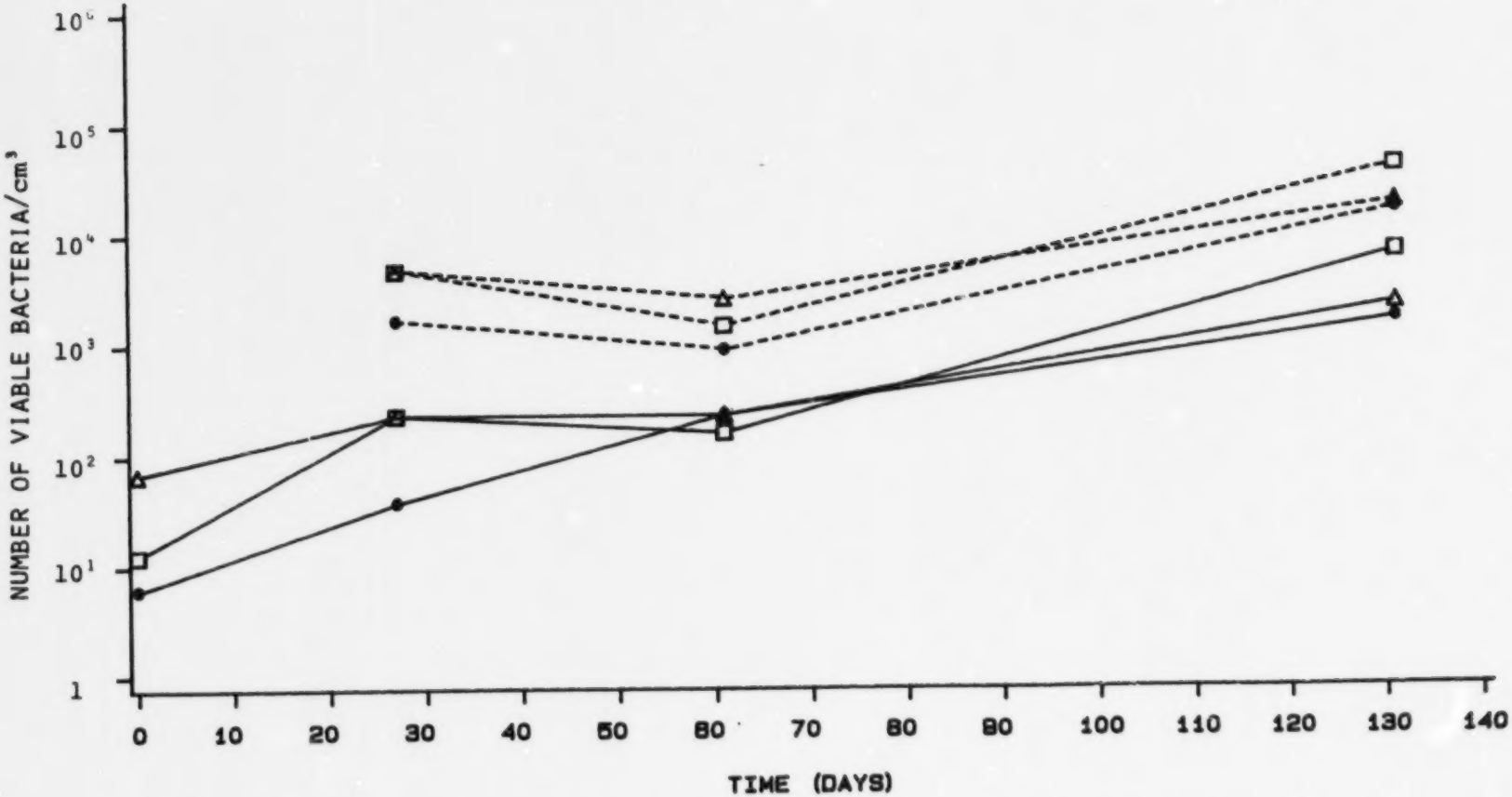

FIGURE 4. INVOLVEMENT OF SULPHATE-REDUCING BACTERIA IN BIOFILM DEVELOPMENT

BACTERIAL NUMBERS IN BIOFILM (-----) AND LAKEWATER (———) IN BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (●), NANTICOKE TGS (□), AND PICKERING NGS (△).

(approximately 4 x 10^2 to 4 x 10^3 cells/cm^3). However, anaerobic sulphate reducers do not occur in large numbers freely suspended in the water, but are active in the sediment layers where oxygen levels are much reduced. There is a relationship between these bacteria and sulphate levels. In a freshwater lake with little sulphate (3 mg/L), no sulphate-reducing bacteria were found in the water and only 1 cell/g sediment at the lake bottom (Kuznetsov, 1970). In contrast, a lake with a high sulphate concentration (2.9 mg/L in surface water to 89 mg/L at a depth of 9 metres) was found to contain 4 to 7.5 cells/cm^3 in the hypolimnion and 2 x 10^3 cells/g in the sediment (Kuznetsov, 1970). Analysis of service water from Pickering and Bruce NGS showed sulphate to be present at concentrations of 30 mg/L and 16 mg/L respectively. There were ten times as many bacteria in the Pickering water, which is consistent with the expected trend, but this was not completely manifested in the biofilm results. Although the greatest number of sulphate reducers was found in the Pickering biofilm at 27 and 61 days, Nanticoke contained more of these bacteria at 131 days. Specific substrate concentration appears to have some effect on the microbial content of water and biofilm.

There were small but significant differences between biofilm and water values in most cases (Appendix C). Numbers in the developed biofilm ranged from 1.68 x 10^4 cells/cm^3 for Bruce to 4.35 x 10^4 cells/cm^3 for Nanticoke. It is difficult to determine unequivocally whether this number of sulphate-reducing bacteria has the potential to cause corrosion. However, analysis of deposits taken from metal surfaces that were pitted can give some idea of the numbers that exist in close proximity to corrosion areas. Sulphate reducers were found in a severely pitted process feedwater line at Bruce Heavy Water Plant at levels of 4.9 x 10^3-1.6 x 10^5 cells/g deposit (Ontario Hydro, 1983 e), and in a failed moderator heat exchanger at Pickering NGS 'B' in greater numbers of 2.4 x 10^4-3.7 x 10^5 cells/g (Ontario Hydro, 1983 b), where failure was partially attributed to bacterial corrosion. Therefore it appears that sulphate reducers were sufficiently numerous in the experimental biofilms for microbiological corrosion processes to occur.

In Figure 5 it can be seen that there is a more pronounced separation of fungal numbers between both biofilm-water composition and between lake types. The pattern of parallels between biofilm and water composition is apparent. Over the 131 day period the rate of increase in attached fungal numbers was greatest in the initial 27 days, followed by a plateau for Bruce and Pickering, during which time there was a marked increase in biofilm numbers for Nanticoke. There was a further increase in both phases for all bioreactors between 61 and 131 days.

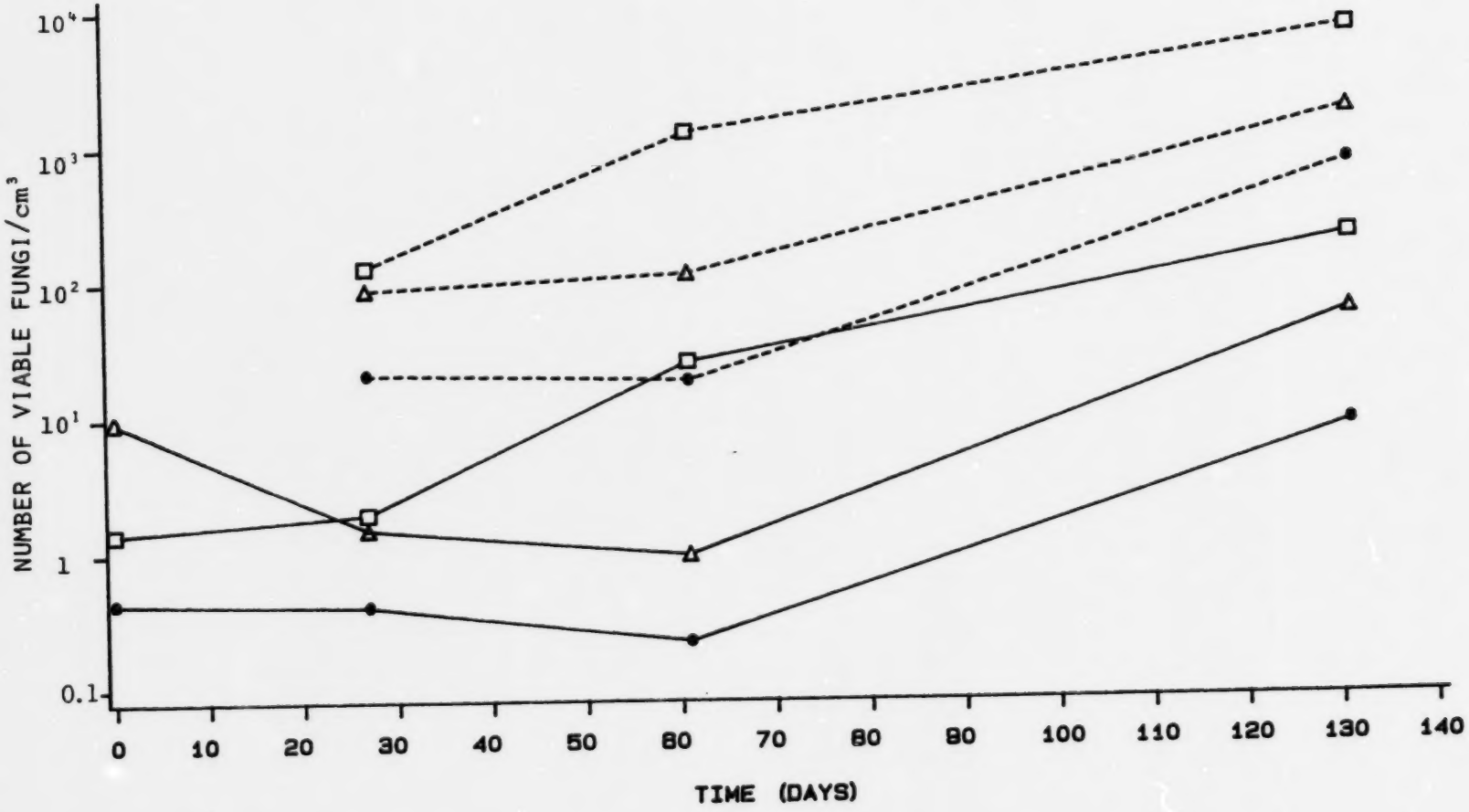

FIGURE 5. INVOLVEMENT OF FUNGI IN BIOFILM DEVELOPMENT.

FUNGAL NUMBERS IN BIOFILM (-----) AND LAKEWATER (———) IN BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (●), NANTICOKE TGS (□), AND PICKERING NGS (△).

The difference between biofilm and water values was statistically significant for all bioreactor types at all points sampled (Appendix C). The ratio of biofilm:water produced numbers that were more consistent than had been observed for the actinomycetes, aerobic heterotrophs and ammonifying bacteria, with values ranging from 31 to 122 times more fungi in the biofilm than in the water.

Despite the clear difference in biofilm - water trends for fungi, this group was not numerically important. However, in the early stages of biofilm development fungi were more efficient at attachment and colonization of metal wafers relative to their available numbers in the water, but only reached maximum counts of 7.1×10^3 cells/cm^3. The oxygenated conditions and temperature around ≈20°C would have favoured fungi, although the alkaline pH would not. Fungi are rarely numerically dominant in balanced ecosystems and so their relatively low numbers in the bioreactors was not unexpected.

Results for the pseudomonads, as shown in Figure 6, reveal a consistent pattern. After the initial rapid increase in numbers attached to the metal surface there was a more gradual rise with no indication of a stable state being attained. The water composition for all lake water types was similar, but while this was the case for Bruce and Nanticoke biofilms, the Pickering biofilm contained more pseudomonads irrespective of the water content. At the end of the test the number of pseudomonads in the biofilm ranged from 5.0×10^6 cells/cm^3 for Bruce up to 2.2×10^7 cells/cm^3 for Pickering. This indicates a well developed biofilm composed of actively metabolizing organisms, particularly as the numbers were still increasing.

Examination of the statistical analysis (Appendix C) revealed that in all instances, for all the bioreactors, there were detectable differences between numbers of pseudomonads in the biofilm and water. The biofilm values were 5 to 61 times greater than the water values, with differences increasing with time.

In summary, all the microbial groups studied preferentially attached to stainless steel wafers. Comparison with other work is difficult as few researchers have used unadulterated lake water, but have chosen to inoculate the system with cultures of potential colonizers. In general the number of micro-organisms comprising the biofilm was less than the level obtained by Tarbuck and Wyborn (1980) and greater than that reported by Pope and co-workers (1982). The initial total number of potential colonizers was low 2.6×10^3 cells/cm^3 to 1.65×10^4 cells/cm^3 for Bruce and Nanticoke, respectively, when compared to the number of respiring bacteria enumerated in the fall, in Lake

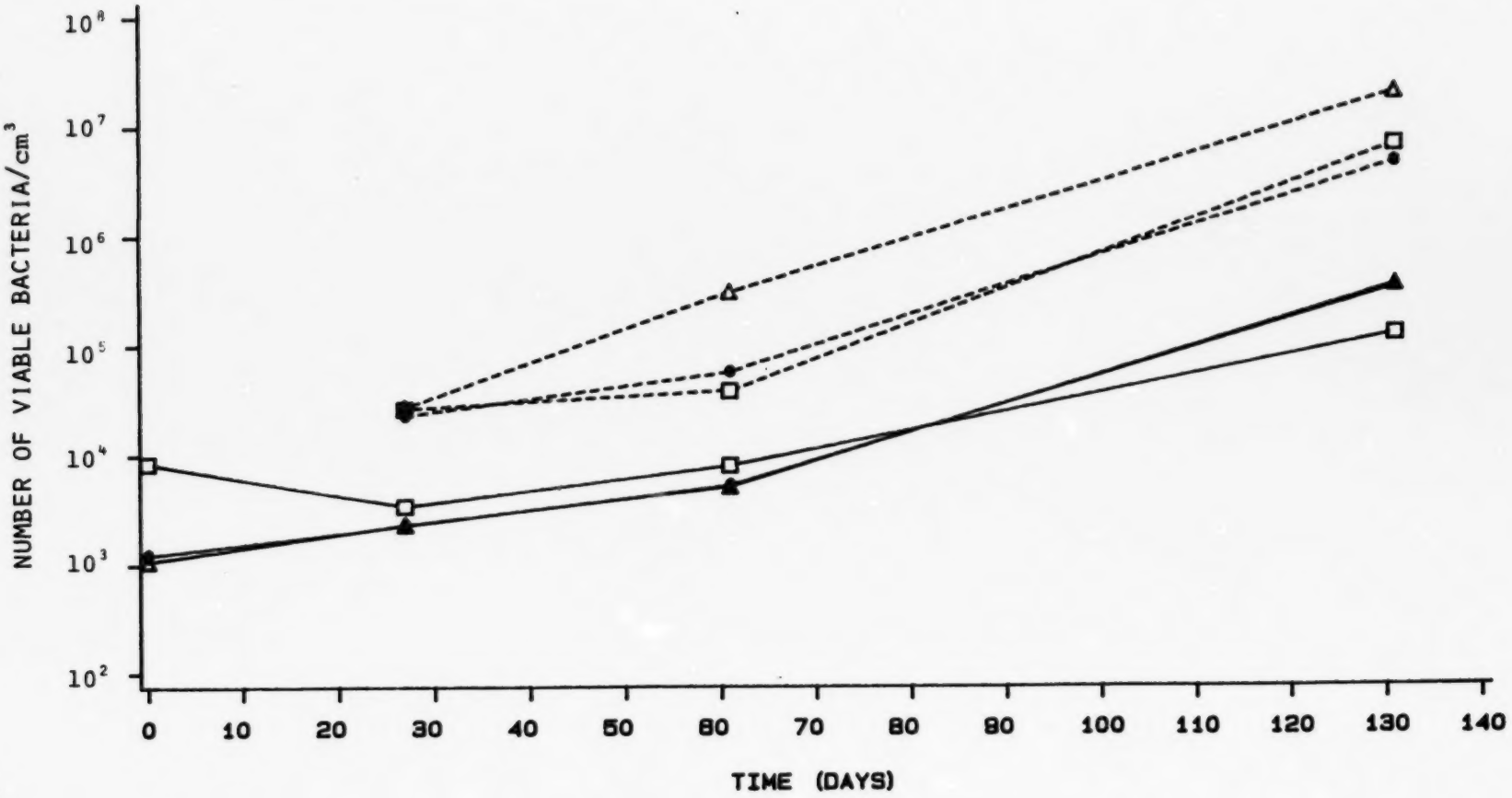

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FIGURE 6. INVOLVEMENT OF PSEUDOMONADS IN BIOFILM DEVELOPMENT.

BACTERIAL NUMBERS IN BIOFILM (-----) AND LAKEWATER (———) IN BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (●), NANTICOKE TGS (□), AND PICKERING NGS (△).

Huron (4.6×10^4 cells/cm^3) and Lake Erie (8.2×10^4 cells/cm^3) (Rao et al, 1981). These low levels may have limited biofilm development to some extent. However, there was rapid early attachment in less than 27 days and further development, probably due to growth rather than attachment, up to the 131 day limit of the experiment without a plateau being attained.

The patterns for biofilm development and water composition were similar and this was attributed to sloughing of the biofilm, with an increasing incidence of aggregates of biological material suspended in the water with increasing time. These aggregates would give rise to a large number of colonies and hence elevate the results obtained for the water samples. Re-entrainment, or sloughing, of attached biomass has been recognized as a phase of biofouling (Characklis, 1980) and is attributed to oxygen/nutrient depletion caused by reduced diffusion in well developed biofilms and water velocity fluctuations (Marshall and Baier, 1981). However in the closed loop test system re-entrainment would result in the biofilm dictating water composition, except in the early stages of microbial attachment. This somewhat artificial system would tend to equalize the biofilm and water values and the partial nutrient limitation would tend to hinder biofilm development. Therefore, it is notable that in almost every instance microbial numbers in the biofilm were significantly greater than in the surrounding water.

There is not a consensus as to why microbes attach to surfaces but is widely accepted that attachment stimulates growth. This may be due to available surface area, increased nutrient concentration at the solid-water interface or more favourable pH, O_2 and CO_2 gradients (Paerl, 1980). In a study of microbial heterotrophic activity it was found that naturally occurring epilithic populations were as much as 8 to 26 times more active per cell than planktonic populations (Ladd et al, 1979). In the light of these studies it would seem reasonable that the natural planktonic microbial populations in the three lake water types examined in this work, would demonstrate enhanced growth and development at the stainless steel-water interface.

3.2 Microbial Speciation in Biofilm Development

To ascertain the importance of microbial speciation in the processes of biofilm attachment and development, the number for each microbial group was expressed as a proportion of the total number for one bioreactor at a specific time in either the solid or water phase and the values are presented in the form of a histogram. Proportions of microbial groups can be compared between bioreactors at a set time point, or the trends within a

microbial group over time can also be compared. To ascertain whether differences in proportion were statistically significant ANOVAs were performed with the cooling water source or time as the variable. Proportions for both water and solid phases can be found in Appendix D (Table 1), while the graphical presentation for water is shown in Figure 7 and for biofilms in Figure 8.

Examination of the microbial composition of the lake water in the three bioreactors, shown in Figure 7, reveals distinct differences in proportion of microbial species present. Initially, the water in all reactors was composed mainly of actinomycetes, aerobic heterotrophs, ammonifiers and pseudomonads, with predominance of each group varying between bioreactors. Statistical analysis showed that actinomycetes dominated in the Bruce water, while water in the Nanticoke and Pickering systems contained significantly more aerobic heterotrophs. At this stage the ammonifiers were also important, with Nanticoke possessing significantly less and Pickering significantly more ammonifiers than the Bruce lake water. The pseudomonads represented a greater proportion of the total viable count than the ammonifiers, but were not present in detectably different numbers in the three bioreactors.

After 61 days the aerobic heterotrophs dominated the water composition and in response to this the proportion of all the other groups of organisms were smaller. The proportion of aerobic heterotrophs in the three bioreactors was not significantly different. However, within the second most numerically important group - the actinomycetes, the value for Bruce was statistically greater than for Nanticoke and Pickering.

By 131 days the dominant microbial groups, actinomycetes aerobic heterotrophs and pseudomonads were present in similar proportions. Within these groups no statistical differences were demonstrated between the three bioreactors. However, within the small ammonifying bacterial group a difference was registered with Nanticoke classified as lower and Pickering as greater than the value for Bruce. In conclusion, the water composition was dominated by actinomycetes in the Bruce bioreactor at the early stages, while aerobic heterotrophs predominated in Nanticoke and Pickering up to 61 days. As the system aged the microbial population became more evenly distributed among the actinomycete, aerobic heterotroph and pseudomonad groups.

Data for comparison are scarce, however, in a major review of the microbial composition of cooling waters a list of prevalent organisms was given (McCoy, 1980). The source of water, the methods of analysis and actual numbers were not given, but the

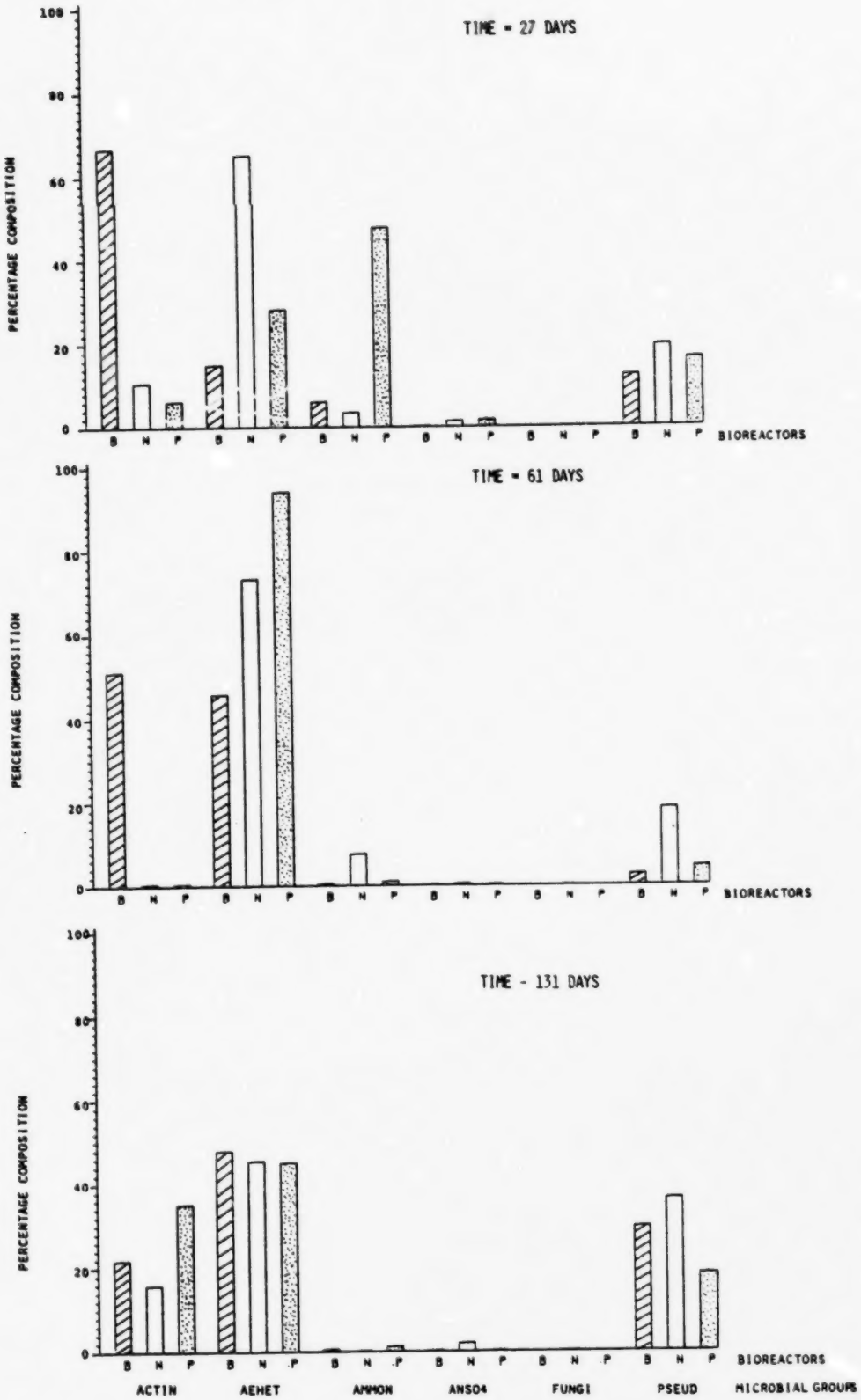

FIGURE 7. VARIATION IN PROPORTION OF MICRO-ORGANISMS PRESENT IN THREE DIFFERENT SOURCES OF COOLING WATER.

BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (B), NANTICOKE TGS (N) AND PICKERING NGS (P). PERCENTAGE COMPOSITION OF WATER FOR MICROBIAL GROUPS - ACTINOMYCETES (ACTIN), AEROBIC HETEROTROPHS (AEHET), AMMONIFIERS (AMMON), ANAEROBIC SULPHATE REDUCERS ($ANSO_4$), FUNGI, PSEUDOMONADS (PSEUD).

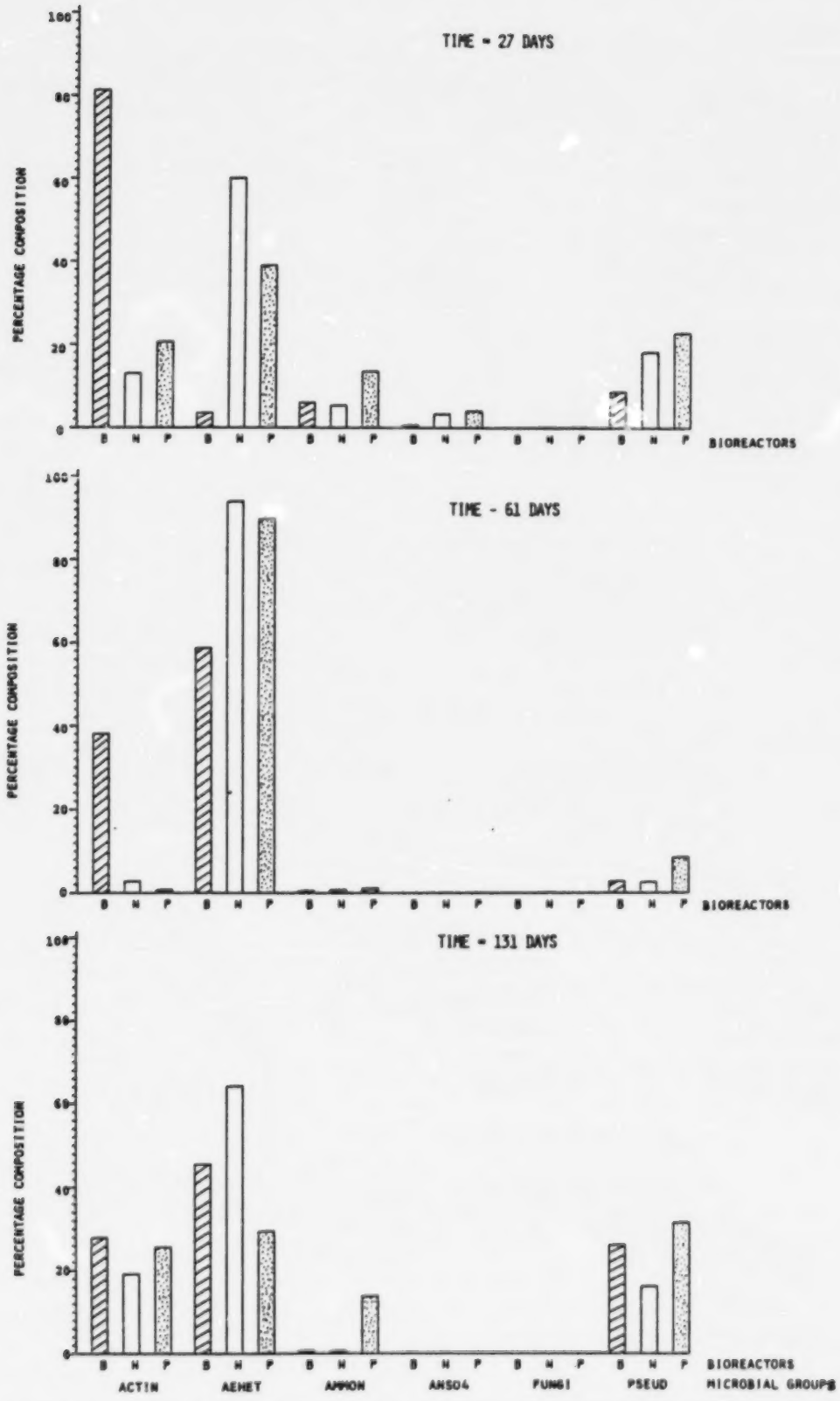

FIGURE 8. VARIATION IN MICROBIAL COMPOSITION OF BIOFILMS ON STAINLESS STEEL WAFERS SUSPENDED IN THREE DIFFERENT SOURCES OF COOLING WATER.

BIOREACTORS CONTAINING COOLING WATER FROM BRUCE NGS (B), HANTICOKE TGS (N) AND PICKERING NGS (P), PERCENTAGE COMPOSITION OF BIOFILM FOR MICROBIAL GROUPS - ACTINOMYCETES (ACTIN), AEROBIC HETEROTROPHS (AEHET), AMMONIFIERS (AMMON), ANAEROBIC SULPHATE REDUCERS (ANSO4), FUNGI, PSEUDOMONADS (PSEUD).

pseudomonads were cited as the major group, followed by the Aerobacter genus, then members of the genus Flavobacterium, sulphate-reducers and anaerobic Bacillus species (McCoy, 1980). Although there is some overlap in the enumeration of aerobic heterotrophs and pseudomonads, this latter group was not dominant in the cooling water examined in this study. The Aerobacter genus, which is no longer an accepted classification (Buchanan and Gibbons, 1974), and Flavobacterium genus fall into the aerobic heterotrophic category and so would be in agreement with the work presented herein. Interestingly McCoy found the sulphate-reducers to be the fourth most prevalent group, which was certainly not the case in this study.

In Figure 8 at 27 days the predominant groups in the biofilm were the same as the water, that is, actinomycetes, aerobic heterotrophs, ammonifiers and pseudomonads. However, within each of these groups the relative numerical importance varied between bioreactors. The proportion of the biofilm composed of actinomycetes was significantly greater in Bruce than in Nanticoke and Pickering. Not surprisingly the reverse is true in the other dominant group, the aerobic heterotrophs, where the value for Bruce was considerably less than Nanticoke and Pickering. The number of ammonifiers and pseudomonads could not be differentiated between bioreactors on the basis of the analysis used here.

At 61 days some changes in biofilm composition had occurred. The only major groups recorded were actinomycetes and aerobic heterotrophs. Actinomycetes were still a statistically important group in the Bruce bioreactor, whereas Pickering was recorded as having an exceptionally low proportion. In the aerobic heterotroph group, although Bruce appeared to have a lower proportion of these bacteria compared to the other two bioreactors, the difference was not statistically significant. The fungal group was not important numerically, however, a statistical comparison of proportions revealed that the value for Nanticoke was exceptionally high and for Bruce very low.

The relative significance of the microbial group proportions altered again at 131 days and followed the same pattern observed for water composition. The proportions of the three major groups, actinomycetes, aerobic heterotrophs and pseudomonads could not be differentiated statistically. There were differences in proportion between bioreactors for the less numerically important group. The proportion of fungi was significantly greater in the Nanticoke bioreactor than in the other two bioreactors, whereas for the ammonifiers Pickering possessed statistically greater and Nanticoke statistically lower proportions than the Bruce biofilm.

Analysis of the relative proportions over time within each microbial group revealed interesting trends in biofilm composition as the biofouling layer developed. In many instances the differences apparent from comparing the histograms vertically are supported by statistical analysis. In the Bruce bioreactor the proportion of actinomycetes was high but the variation in proportion with time was within the estimated variance. Aerobic heterotrophs were recorded as significantly low at 27 days and high at 61 days, whereas pseudomonads were shown to be statistically greater than expected at 131 days.

The pattern for the Nanticoke system showed that the proportion of aerobic heterotrophs was significantly low at 27 days in relation to the higher values as the biofilm developed. In contrast the anaerobic sulphate reducers and fungi had proportions greater than expected at 27 and 61 days respectively, whereas actinomycetes increased in importance by the end of the experiment.

The Pickering data revealed large fluctuations in microbial population with time that were greater than experimental error. The proportion of aerobic heterotrophs was less than predicted at 27 days and greater at 61 days, indicating a major change in biofilm composition with respect to these bacteria. Comparison between the proportion of actinomycetes in the biofilm revealed a significantly low level at 61 days which was reversed at 131 days. Anaerobic sulphate-reducing bacteria had an unusually high count in the biofilm at 27 days that decreased progressively to produce a statistically lower proportion at 131 days.

Overall trends, which can be seen in Figure 8, indicate that the biofilm is composed predominantly of aerobic heterotrophs, actinomycetes, pseudomonads, and to some extent ammonifiers. However, the predominance of these groups in the biofilm varies between bioreactors and with stage of biofilm development.

Actinomycetes may be predisposed to attachment because of their filamentous growth but their relative importance in the early stages of biofilm development in the Bruce reactor may be due to the nutrient status of Lake Huron. This lake is oligotrophic and actinomycetes are oligocarbophilic, to the extent that they will not grow in the presence of high concentrations of organic compounds (Kuznetsov, 1970). Under such conditions it is possible that actinomycetes grow better than aerobic heterotrophic bacteria which favour higher concentrations of organics. As nutrient levels increase, through concentration by adsorption and growth of microbes at the metal surface, then aerobic heterotrophs would predominate.

This hypothesis was not supported by an increase in actinomycete numbers in response to decreased aerobic heterotroph proportion in the more nutrient rich lake waters of Ontario and Erie. Actinomycetes are shown to be nutritionally versatile and may produce extracellular enzymes that permit the utilization of polysaccharides (Buchanan and Gibbons, 1974). It is possible that as the proportion of slime-producing pseudomonads increased, growth of actinomycetes was stimulated through the increased availability of this specific nutrient source. At this stage of biofilm development actinomycete filaments may be involved in the binding together of the attached biomass. It has been observed that developed biofilms were largely filamentous in the presence of low levels of glucose, and with increased glucose concentrations the biofilm structure shifted to a non-filamentous matrix (Characklis et al, 1981). Although this work used nutrient additives and a controlled microbial population it appears to be supportive of the "binding" role which actinomycetes may play under suitable nutrient conditions.

The aerobic heterotrophic bacteria constituted the major group in the biofilm in all bioreactors, particularly at 61 days, ranging in proportion from 59% (Bruce) to 94% (Nanticoke). This nutritionally versatile, highly diverse group can be classified as opportunistic and so as they were present in the water in large numbers they were the most effective at taking advantage of the metal surface on which to grow. The wafer surfaces concentrate nutrients by adsorption, thereby enhancing microbial growth. This behaviour implies that initiation of a biofouling layer is reliant upon the presence of large numbers of fast growing micro-organisms. Furthermore, this provides supportive evidence that the mechanism of microbial adhesion is strongly affected by the number of cell-surface contacts, and so the total number of microbes and cooling water velocity would be important. It has been cited that bacteria which are first to attach and colonize the water-solid interface, would have a propensity for attachment and possess a faster growth rate than the surrounding microbes (Corpe and Winters, 1980). In a detailed analysis of biofilm composition aerobic heterotrophs were found to be the dominant group (Tarbuck and Wyborn, 1980). These results concur with the work reported here.

Pseudomonads were isolated from the biofilm in relatively large numbers and this can be seen in Figure 8. They are thought to be an important biofilm constituent because of their ability to secrete an abundance of extracellular polymers (Marshall and Baier, 1981; Corpe, 1980). Furthermore, this secretion of slime-like material has been associated with development of a biofouling matrix in the later stages of biofilm development (Eaton et al, 1980; Little and Lavoie, 1980). In contrast, microbes encased in a polysaccharide capsule were selected for

study on the basis that they would be more efficient colonizers of a model heat exchange system, rather than being involved in more mature biofilm development (Harty and Bott, 1981). In this study the pseudomonads were probably more important in the latter stages as their numbers increased gradually with time. The presence of pseudomonads may have a significant effect with regard to corrosion. Certain strains of the Pseudomonas fluorescens-putida group produce a siderophore - a component which binds iron and is believed to enhance cellular uptake (Dixon, 1984). This scavenging of iron may well accelerate corrosion processes.

The proportion of ammonifying bacteria in the biofilm was small compared to the three groups discussed previously and so it is difficult to assess their importance. This comment is also valid for the anaerobic sulphate-reducing bacteria, which comprised a very small portion of the biofilm. This group may not be important in biofilm development per se, but may still have an impact on corrosion processes. Even low numbers of sulphate reducers have been shown to corrode metals (Ontario Hydro, 1983 e) and as an anaerobic environment can be created by a biofilm only ten cells deep (Coulter and Russell, 1976), these bacteria should be able to function effectively within the sort of bulky biofilm obtained in this work.

The final group, the fungi, showed considerable variation in proportion, but were present in such relatively low numbers that any interpretation is difficult. Fungi may be involved in binding together the attached biomass, where this can be performed by a relatively few filaments and so numerical dominance is not required, but supportive evidence for this concept could not be found.

This aspect of interpretation, whether the significance of a contribution by a microbial group to biofilm development should be allocated solely on the basis of numerical dominance, is problematical. It can be said that certain micro-organisms do not have to be present in large numbers to be effective, such as the involvement of sulphate reducers in corrosion. However, some properties of the biofilm, slime content, compactness, will be mainly dictated by the nature of the major constituents and so numerical dominance must be considered as an important factor.

There appeared to be many similarities between the proportion of microbes in the biofilm and in the water and so a statistical analysis was performed to determine if this was the case. The values for each group in the biofilm should be a constant multiple of the water value, or at least within the range defined by an estimate of total variability. Any significant differences will be indicated by the ANOVA, the results of which

can be seen in Appendix D (Table 2). Because the variances were not equal for spread plate counts and MPN derived values, the ANOVA was not strictly appropriate and hence only an approximate test was constructed. The results of the test indicate that no statistically significant differences were found except for the Nanticoke bioreactor at 61 and 131 days and for Pickering at 131 days. The extreme high and low values for both the bioreactors at 131 days are due to ammonifying and sulphate-reducing bacteria counts, which were obtained using the MPN method. Therefore, the statistically demonstrated difference in these two cases is probably spurious, with the only true difference recorded for actinomycetes in the Nanticoke reactor at 61 days. Hence water and biofilm composition was similar, which is not unexpected when the effect of biofilm re-entrainment into the closed loop system is taken into consideration.

It was concluded that within the three bioreactors specific microbial groups predominated to differing extents in biofilm development. Furthermore, the relative importance of a microbial group varied at different stages of development. Actinomycetes played an important role in metal surface colonization and early growth in the Bruce system, which was not observed in the other two bioreactors. In fact the pattern of microbial species involvement in biofilm development was similar for Nanticoke and Pickering. As the test systems aged the relative importance of groups changed and became alike for all bioreactor types, with actinomycetes, aerobic heterotrophs and pseudomonads being numerically dominant. The composition of water and biofilm was similar at each stage but the initial and final microbial populations for both phases were different. This succession of predominance highlights the dynamic nature of biofilms and explains why microbial speciation within the biofilms is not consistent with time. Therefore, it would appear that analysis of water composition may allow prediction of the nature of the biofilm at a certain stage of development, but not for the entire process.

3.3 Biofouling Potential

In this section the final objectives will be addressed. To ascertain whether the cooling water sources possess differing capacities to cause biofouling, it must be determined if the water and biofilm composition varies between the three lake water types. As it has been noted that water content was largely a function of biofilm composition after the initial stage due to sloughing of the film, only initial water values have been included for comparison. The three bioreactors were compared by conducting a modified ANOVA on each phase (biofilm and water) at each time point for a specific microbial group. After each ANOVA a Fishers' Least Significant Difference (FLSD)

was calculated to determine the source of the difference. If there are differences, then could the biofilm composition have been predicted based on the initial water composition?

The statistical comparison between the three bioreactors for actinomycetes is presented in Table 1. In the water phase initial actinomycetes numbers were greatest in the Nanticoke bioreactor, with no detectable difference between Bruce and Pickering values. To predict biofouling potential with regard to actinomycete numbers, the order of dominance of bioreactors in the initial water sample must be compared with the biofilm composition, with emphasis on the final value. The Nanticoke bioreactor contained the most actinomycetes initially but this did not occur in the biofilm at any stage. Furthermore, the importance of actinomycetes in Bruce biofilm development at 27 and 61 days could not have been predicted by microbiological analysis of the original water constituents.

TABLE 1

COMPARISON OF NUMBERS OF ACTINOMYCETES IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	$\log_e$ Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] ($P = 0.05$)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	5.75	7.66	5.90	<0.0001	0.88	B P, N
BF	27	12.34	9.88	10.15	<0.0001	0.84	N P, B
BF	61	13.57	10.63	10.24	<0.0001	0.88	P N, B
BF	131	15.49	15.99	16.71	0.023	0.87	B N, P

[1] Derived by modified analysis of variance

[2] Bioreactors are similar if the difference in $\log_e$ mean numbers is <Fishers LSD

The order of predominance of aerobic heterotrophic bacteria in solid and water phases at different times was statistically tested and the results can be seen in Table 2. Prediction of the potential of aerobic heterotrophic bacteria to cause biofouling is based on whether high numbers in the original water sample are also found in the developed biofilm. In the initial water Bruce contained the least aerobic heterotrophs, while Pickering and Nanticoke possessed similar but greater numbers of these bacteria. This exact pattern was manifested in the biofilm at 27 and 131 days. Therefore, over the time span tested, an analysis of aerobic heterotrophic bacterial composition of the cooling water would have allowed prediction of the extent of biofouling caused by this group. However, it should be noted that the order of predominance of these bacteria in the biofilm was not consistent throughout the test, so that had the experiment been concluded after 61 days then this trend would not have been so easily detected.

TABLE 2

COMPARISON OF NUMBERS OF AEROBIC HETEROTROPHIC BACTERIA IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	$\log_e$ Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] ($P = 0.05$)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	6.65	8.60	7.78	<0.0001	0.85	B P N
BF	27	9.18	11.40	10.78	<0.0001	0.86	B P N
BF	61	14.00	14.17	14.99	0.060	0.88	B N P
BF	131	15.97	17.21	16.85	0.015	0.86	B P N

[1] Derived by modified analysis of variance

[2] Bioreactors are similar if the difference in $\log_e$ mean numbers is <Fishers LSD

The results and statistical comparisons for ammonifying bacteria in different bioreactors are shown in Table 3. There were few detectable differences for ammonifying bacteria between the bioreactor types probably because of the large inherent analytical error associated with the MPN technique.

TABLE 3

COMPARISON OF NUMBERS OF AMMONIFYING BACTERIA IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	Log_e Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] ($P = 0.05$)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	5.48	5.48	6.65	0.473	1.98	B N P
BF	27	9.73	8.99	9.73	0.685	1.91	B P N
BF	61	9.34	9.32	10.68	0.243	1.82	N P B
BF	131	11.65	12.50	16.09	<0.0001	1.88	B N, P

[1] Derived by modified analysis of variance

[2] Bioreactors are similar if the difference in log_e mean numbers is <Fishers LSD

The numbers of ammonifiers in the original water appeared to range from the least in Bruce to the greatest in Pickering, but the differences could not be demonstrated statistically. This order of predominance was observed in the final biofilm composition with Pickering now demonstrating a distinct separation. Hence an element of prediction appears to be possible, but greater resolution would require the use of a different enumeration technique.

The data for anaerobic sulphate-reducing bacteria is presented in Table 4. The same problem with identifying trends for ammonifying bacteria was found for sulphate reducers. The greater variability associated with the MPN method can be seen in the higher value calculated for Fisher's Least Significant Difference, as compared to the value obtained when a spread plate method was used for quantification. Numbers of sulphate reducers in the water analyzed prior to experimentation revealed that Bruce and Nanticoke bioreactors could not be differentiated, but contained fewer bacteria than Pickering. Although the relative numbers in the biofilms were the same at 27 and 61 days, but not at 131 days, the difference between bioreactors could not be demonstrated statistically. Therefore, it was

concluded that the biofouling potential of sulphate-reducing bacteria could not be predicted in this experiment, and probably not in future work if the same method of quantification was used.

TABLE 4

COMPARISON OF NUMBERS OF SULPHATE-REDUCING BACTERIA IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	$\log_e$ Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] ($P = 0.05$)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	1.83	2.57	4.25	0.042	1.93	B N P
BF	27	7.43	8.50	8.50	0.491	2.04	B N P
BF	61	6.81	7.33	7.90	0.484	1.77	B N P
BF	131	9.73	10.68	9.90	0.558	1.85	B P N

[1] Derived by modified analysis of variance
[2] Bioreactors are similar if the difference in $\log_e$ mean numbers is <Fishers LSD

The differences in composition of biofilm and water with respect to fungi in the three bioreactors are shown in Table 5. In the water analysis the numbers of fungi were greatest in the Pickering bioreactor, with Bruce and Nanticoke possessing similar and fewer numbers of microbes. In the biofilm the lowest values were observed for Bruce at all stages of development. Initially, the composition of the Pickering and Nanticoke biofilms could not be differentiated, but from 61 days onwards Nanticoke had the greatest number of fungi. This trend in biofilm composition was the same throughout the test, but did not tie in with water composition, where the smallest values for Bruce were consistent but results for Nanticoke and Pickering were not. Hence, it was concluded that some indication of biofilm constituents could be obtained from analysis of fungal numbers in the lake water.

TABLE 5

COMPARISON OF NUMBERS OF FUNGI IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	Log_e Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] ($P = 0.05$)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	-0.87	0.35	2.26	<0.0001	0.95	B N P
BF	27	3.05	4.87	4.48	<0.0001	0.89	B P N
BF	61	2.92	7.17	4.75	<0.0001	0.83	B P N
BF	131	6.59	8.86	7.47	<0.0001	0.84	B P N

[1] Derived by modified analysis of variance

[2] Bioreactors are similar if the difference in log_e mean numbers is <Fishers LSD

The results for the differences in numbers of pseudomonads between bioreactors are set out in Table 6. Statistical analysis revealed that pseudomonads were present in greater numbers in the Nanticoke bioreactor water, and to a lesser, but similar, extent in the Pickering and Bruce systems. This order of numerical dominance was not seen in the biofilm, where the similar values for Bruce and Nanticoke were exceeded by Pickering at 61 and 131 days. Predominance of pseudomonads in the Pickering biofilm was not indicated in the original water composition and so prediction of this microbial group's involvement in biofouling would not have been possible from lake water analysis. In fact the low number of pseudomonads in the Pickering service water was unexpected because of the relative importance of these bacteria in biofouling deposits taken from a failed moderator HX at Pickering NGS had been noticed, when compared to deposits taken from a moderator HX at Bruce NGS (Ontario Hydro, 1983 b and 1983 c).

TABLE 6

COMPARISON OF NUMBERS OF PSEUDOMONADS IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	Log_e Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] (P = 0.05)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	7.14	9.06	6.99	<0.0001	0.86	P B, N
BF	27	10.08	10.20	10.24	0.9271	0.87	B N P
BF	61	10.94	10.59	12.64	<0.0001	0.86	N B, P
BF	131	15.41	15.83	16.92	<0.0001	0.87	B N, P

[1] Derived by modified analysis of variance

[2] Bioreactors are similar if the difference in log_e mean numbers is <Fishers LSD

The total number of micro-organisms in the three bioreactors was compared and ranked, the results of which are shown in Table 7. In ascending order the total number of microbes present in the water were Bruce, Pickering and Nanticoke. This pattern was not manifested in the biofilm composition in the early stages of development, but at 131 days Bruce had significantly fewer organisms than Nanticoke and Pickering which could not be differentiated. It would appear that prediction of biofouling potential using total numbers is not possible in the early stages of biofilm development, but has some merit as the biofilm composition begins to stabilize.

The pre-test analysis of total numbers in the lake water appears to be consistent with expected values. Bruce NGS service water comes from the oligotrophic Lake Huron, which is scarce of biogenic elements and so will not support extensive microbial growth under normal circumstances. Nanticoke NGS service water may be affected to some extent by two local streams which drain agricultural land and hence could wash fertilizers into Lake Erie, particularly after a storm. The service water at Pickering NGS is likely to contain both high levels of nutrients

TABLE 7

COMPARISON OF TOTAL MICROBIAL NUMBERS IN BIOFILM AND WATER PHASES PRESENT IN THREE SOURCES OF COOLING WATER

Phase Biofilm (BF) Water (W)	Time (Days)	Log_e Number of Microbes per cm^3			Probability of Similarity Between Bioreactors [1] ($P = 0.05$)	Fisher's Least Significant Difference	Bioreactors Grouped According to FLSD [2]
		Bioreactors					
		Bruce B	Nanticoke N	Pickering P			
W	0	7.87	9.71	8.44	<0.0001	0.55	B P N
BF	27	12.55	11.92	11.73	0.019	0.60	P N, B
BF	61	14.54	14.25	15.12	0.072	0.75	N B, P
BF	131	16.78	17.66	18.08	<0.0001	0.53	B, N P

[1] Derived by modified analysis of variance

[2] Bioreactors are similar if the difference in log_e mean numbers is <Fishers LSD

and microbial contaminants from several sources. Generally west to east currents will sweep pollutants from the Toronto shoreline towards the station, there is a major sewage treatment plant to the east of the GS, Duffin Creek empties into this area and there is a good deal of recreational use on the 'A' side of the plant. All of these sources will have an input that varies seasonally and temporally but may still be sufficient to affect the microbiological content of the cooling water. It has been observed, in an experimental system, that the fouling rate was dependent upon the glucose loading rate to the system, with glucose as the only source of carbon for microbial growth (Characklis, 1980). Therefore, the lower total number of microbes in the Bruce biofilm compared to Nanticoke and Pickering is consistent with the relative nutrient status of each of the service water sources.

In conclusion, clear differences in water composition between bioreactors were demonstrated, but these differences were not necessarily observed at all stages of biofilm development. As the biofilm began to stabilize the relationship between water

and biofilm became better defined. The ability to predict the extent of biofouling by a specific microbial group after determining the composition of the cooling water was possible for aerobic heterotrophs, to some extent for ammonifiers and fungi, and not at all for actinomycetes, sulphate-reducing bacteria and pseudomonads. Examination of total microbial numbers in the lake waters allowed some prediction of the extent of biofouling, where this potential may be controlled by nutrient status of the cooling water. On the basis of total numbers of microbes, which as discussed previously is a major if not the only criterion, the cooling water at Bruce NGS had the lowest biofouling potential, while water in the vicinity of Nanticoke and Pickering stations would cause more biofouling.

4.0 CONCLUSIONS

The use of a closed loop experimental system had some limitations in that biofouling development would be enhanced by containment of the sloughed biofilm within the bioreactors and by the low velocity water currents used. Alternatively, the extent of biofouling may have been restricted by a lack of nutrients which would normally have been provided by introduction of fresh water in a once through cooling system. Interpretation of the experimental data will allow extrapolation to operating flow-through systems with respect to biofilm composition and relative biofouling levels in the three lakewater types. There is some limitation in correlating the rate and extent of biofouling in the model with a real life situation. Therefore, it is felt that the test system was sufficiently realistic to provide valuable information.

Examination of the process of biofouling of stainless steel waters suspended in three different sources of cooling water, revealed that there was rapid early attachment of all the microbial groups studied. A comparison between the numbers of micro-organisms in the biofilm attached to the metal and the surrounding water, showed that there was a significantly greater number of organisms in the biofilm for actinomycetes, aerobic heterotrophic bacteria, fungi and pseudomonads at levels of 3 to 400 times greater. Significant differences between biofilm and water levels were also demonstrated for anaerobic sulphate-reducing bacteria and ammonifying bacteria, although the results were less consistent due to the greater variability associated with the methods used to enumerate these groups. It has been established that microbes commonly found in lake water readily biofoul submerged metal surfaces in a closed loop system. This layer of biological material may alter the surface properties of heat exchanger tubes, possibly interfering with off-line chemical cleaning procedures.

The attached biomass, observed using a scanning electron microscope, exhibited patchy, uneven colonization which remained when the biofilm was well developed. This mode of adhesion implied that metal surface energy may be important in the biofouling process. The possible involvement of microbes in corrosion of stainless steel through the creation of differential oxygen concentration cells was also suggested by this type of attachment. The presence of fibrillar material provided visual evidence that biologically produced exopolymers were involved in biofilm development. Qualitative information on biofilm composition by energy dispersive x-ray analysis did not give any consistent results for elements of likely biological origin, although measurements of phosphorous, sulphur and potassium were obtained.

Analysis of the proportions of microbial species involved in biofouling showed that bacterial groups predominated over fungi, with aerobic heterotrophs, actinomycetes, pseudomonads and ammonifiers constituting the major elements in all the systems. However, biofilm composition was different for the cooling water systems tested and the predominance of specific microbial groups changed as the biofilm aged. Actinomycetes were dominant in the early stages of biofilm development in the Bruce system and were prevalent in all systems at 131 days. This indicates that a filamentous morphology may be instrumental in binding the biofilm together. The pattern of microbial species involvement in biofilm development was similar for Nanticoke and Pickering, but all three systems became more alike with increasing time. This succession of predominance highlights the dynamic nature of biofilms and explains why microbial speciation within biofilms is not consistent with time. Therefore, it would appear that analysis of water composition may allow prediction of the nature of the biofilm at a certain stage of development, but not for the entire process.

Clear differences in water composition between the three sources were demonstrated, but these differences were not always apparent in the biofilm composition at all stages of development. The ability to predict the extent of biofouling by a specific microbial group, after determining the composition of the cooling water, was possible for aerobic heterotrophs, to some extent for ammonifiers and fungi and not all for actinomycetes, sulphate-reducing bacteria and pseudomonads. Although the total number of each microbial group may not be the only criterion for assessing severity of biofouling it is an important aspect. Therefore, it is significant that analysis of cooling water for total numbers did allow some prediction of the extent of biofouling that occurred and that this in turn was affected by the nutrient status of the lake water. On this basis it was concluded that cooling water in the vicinity of

Bruce NGS had the lowest biofouling potential, and that greater biofouling could be expected in the Pickering and Nanticoke stations. The prediction of biofouling potential from water composition analysis is complicated by the dynamic nature of biofilm development and probably by seasonal and temporal variations in lake water quality. However, it has been shown that cooling water composition varies dependent upon its source and that this does affect the nature and extent of biofouling, which can, to a limited extent, be predicted.

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APPENDIX A

CULTURE MEDIA

Actinomycetes

soluble starch	10.
casein* (dissolve 0.3 g in 10 cm^3 of 0.1 N NaOH)	0.3 g
K_2HPO_4	2. g
$MgSO_4$ .7 H_2O	0.05 g
KNO_3	2. g
NaCl	2. g
$CaCO_3$	0.02 g
$FeSO_4$ .7 H_2O	0.01 g
agar	20. g
de-ionized water	up to 1000. cm^3

Heat to dissolve. Cool. Adjust pH with HCl to 7.0. Autoclave 115°C for 20 minutes.

Add aseptically 10 cm^3 of cycloheximide solution.

* Source - Difco - vitamin free casein.

Cycloheximide Solution

cycloheximide/acitidione	0.05 g
de-ionized water	10. cm^3

When added to the above medium the cycloheximide will be present at a final concentration of 0.005% w/v.

Pour plates with approximately 20 cm^3/plate to prevent unacceptable loss of medium during prolonged incubation period.

Pseudomonads

polypeptone peptone	20. g
K_2HPO_4	1.5 g
$MgSO_4$ .7 H_2O	1.5 g
agar	14. g
de-ionized water	1000. cm^3
pH 7.2	

Suspend 37 g of the dehydrated medium (BBL) in a litre of de-ionized water. Add 10 cm^3 glycerol and heat with frequent agitation and boil for one minute. Sterilize by autoclaving at 115°C for 20 minutes. Pour plates (≈ 15 cm^3/plate).

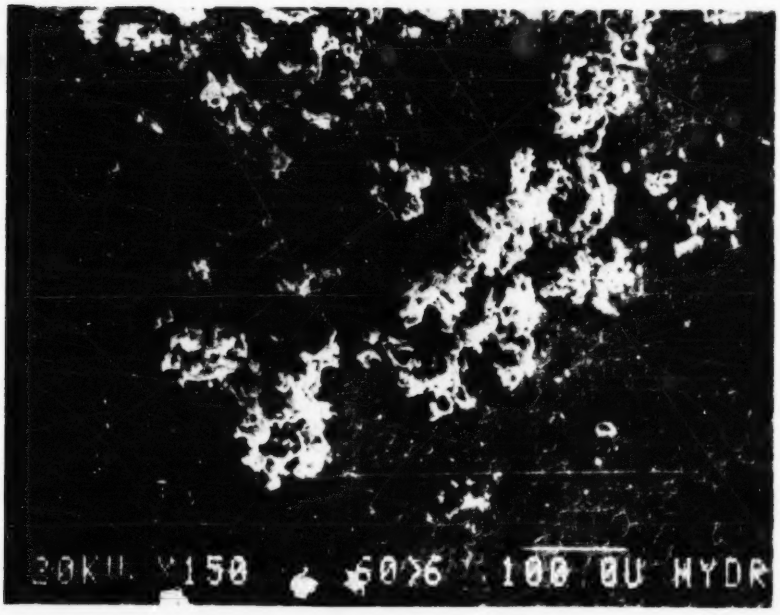
APPENDIX B

SCANNING ELECTRON MICROGRAPHS

Photographs of biofouled stainless steel surfaces obtained using a scanning electron microscope. The network of irregularly crossed lines, which has a mottled appearance, is the bare metal surface. The attached biological material is defined by the pale grey and white patches placed somewhat centrally on the wafers. The clarity of these photographs is better in the originals, which can be supplied upon request.

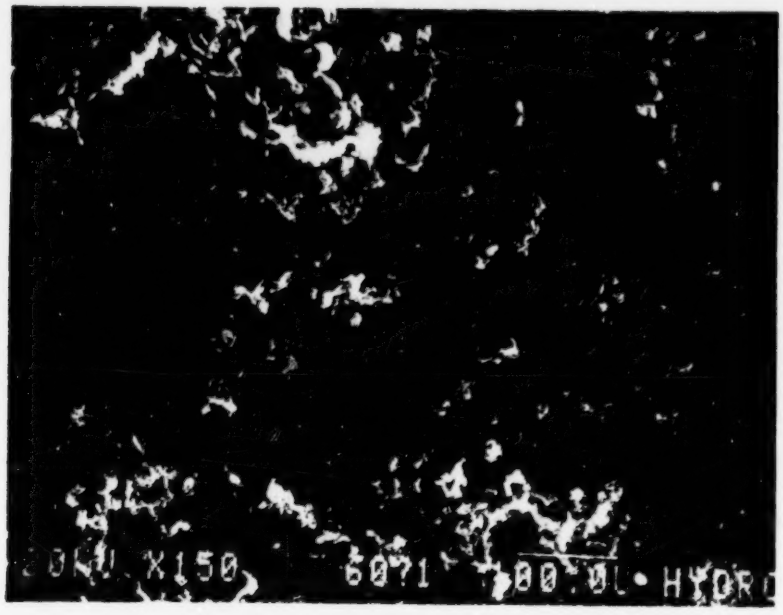
PHOTOGRAPH 1

EARLY STAGES OF BIOFILM ATTACHMENT.
L. HURON - 27 DAYS (x150)



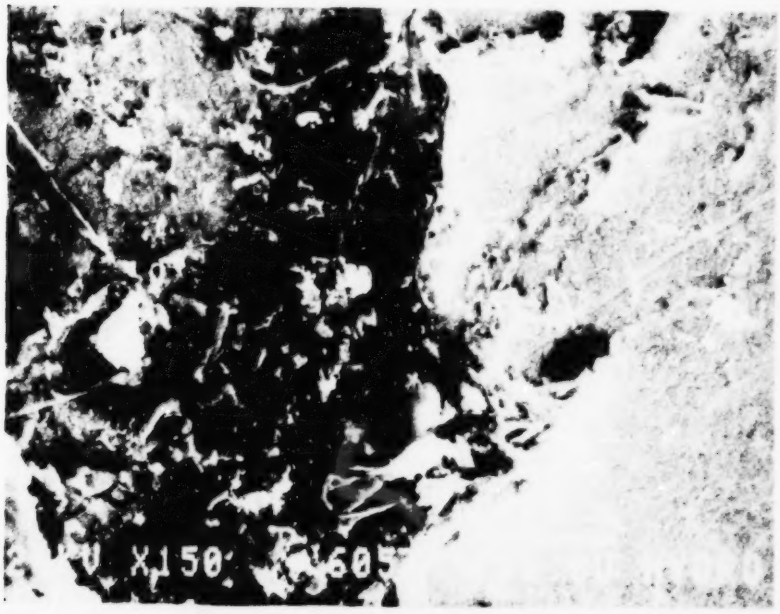
PHOTOGRAPH 2

BIOFILM ATTACHED TO STAINLESS STEEL WAFER.
L. HURON-61 DAYS (x150)



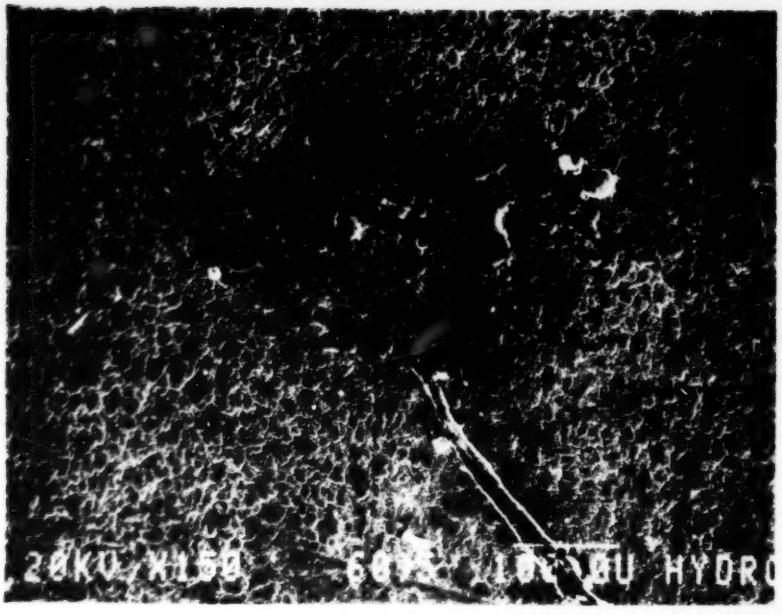
PHOTOGRAPH 3

EARLY STAGES OF BIOFILM ATTACHMENT.
L. ONTARIO - 27DAYS (x 150)



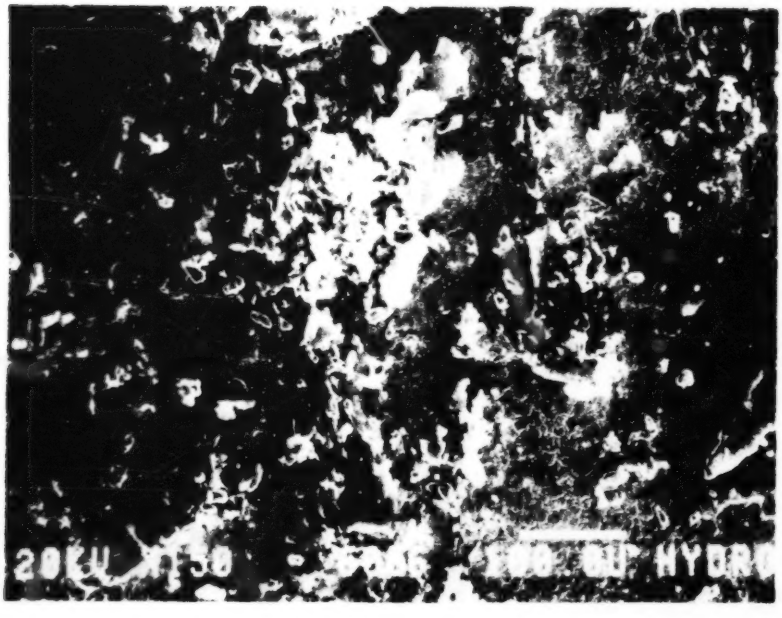
PHOTOGRAPH 4

BIOFILM ATTACHED TO STAINLESS STEEL WAFER.
L. ONTARIO - 61 DAYS (x 150)



PHOTOGRAPH 5

EARLY STAGES OF BIOFILM ATTACHMENT.
L. ERIE - 27 DAYS (x 150)



PHOTOGRAPH 6

BIOFILM ATTACHED TO STAINLESS STEEL WAFER.
L. ERIE - 61 DAYS (x 150)

APPENDIX C

STATISTICS FOR BIOFILM DEVELOPMENT

TABLE 1

ACTINOMYCETES - APPROXIMATE TEST FOR DIFFERENCES BETWEEN LAKEWATER AND BIOFILM

Cooling Water Source	Time (Days)	Microbial Numbers ($\log_e$)		Biofilm: Water Ratio	Z Statistic	Probability of Similarity (P = 0.05)
		Biofilm	Water			
Bruce	27	12.34	9.46	18.	6.75	<0.0001
B	61	13.58	11.56	8.	4.57	<0.0001
B	131	15.50	12.42	22.	7.12	<0.0001
Nanticoke	27	9.88	7.55	10.	5.44	<0.0001
N	61	10.65	5.30	211.	11.85	<0.0001
N	131	16.00	10.96	156.	10.90	<0.0001
Pickering	27	10.15	6.80	29.	7.77	<0.0001
P	61	10.24	6.40	47.	8.28	<0.0001
P	131	16.72	13.45	26.	7.28	<0.0001

TABLE 2

AEROBIC HETEROTROPHS - APPROXIMATE TEST FOR DIFFERENCES BETWEEN LAKEWATER AND BIOFILM

Cooling Water Source	Time (Days)	Microbial Numbers ($\log_e$)		Biofilm: Water Ratio	Z Statistic	Probability of Similarity (P = 0.05)
		Biofilm	Water			
Bruce	27	9.19	7.96	3	2.81	0.005
B	61	14.01	11.45	13	5.93	<0.0001
B	131	15.99	13.21	16	6.31	<0.0001
Nanticoke	27	11.41	9.36	8	4.34	<0.0001
N	61	14.19	10.40	44	8.51	<0.0001
N	131	17.21	12.01	182	12.13	<0.0001
Pickering	27	10.79	8.33	12	5.61	<0.0001
P	61	15.01	11.61	30	7.76	<0.0001
P	131	16.85	13.71	23	7.15	<0.0001

TABLE 3

AMMONIFIERS - APPROXIMATE TEST FOR DIFFERENCES BETWEEN LAKEWATER AND BIOFILM

Cooling Water Source	Time (Days)	Microbial Numbers ($\log_e$)		Biofilm: Water Ratio	Z Statistic	Probability of Similarity (P = 0.05)
		Biofilm	Water			
Bruce	27	9.73	7.05	15	2.76	0.006
B	61	9.34	6.82	13	2.79	0.005
B	131	11.65	8.86	16	2.86	0.004
Nanticoke	27	8.99	6.43	13	2.57	0.01
N	61	9.32	8.13	3	1.31	0.192
N	131	12.50	6.43	433	5.90	<0.0001
Pickering	27	9.73	8.86	2	0.85	0.395
P	61	10.68	7.03	39	3.67	<0.0001
P	131	16.09	10.09	405	6.12	<0.0001

TABLE 4

ANAEROBIC SULPHATE REDUCERS - APPROXIMATE TEST FOR DIFFERENCES BETWEEN LAKEWATER AND BIOFILM

Cooling Water Source	Time (Days)	Microbial Numbers ($\log_e$)		Biofilm: Water Ratio	Z Statistic	Probability of Similarity (P = 0.05)
		Biofilm	Water			
Bruce	27	7.43	3.65	44	3.76	<0.0001
B	61	6.81	5.48	4	1.45	0.146
B	131	9.73	7.43	10	2.59	0.01
Nanticoke	27	8.50	5.48	21	2.94	0.003
N	61	7.33	5.09	9	2.63	0.008
N	131	10.68	8.86	6	1.74	0.082
Pickering	27	8.55	5.48	21	2.94	0.003
P	61	7.90	5.48	11	2.41	0.016
P	131	9.90	7.78	8	2.37	0.018

TABLE 5

FUNGI - APPROXIMATE TEST FOR DIFFERENCES BETWEEN LAKEWATER AND BIOFILM

Cooling Water Source	Time (Days)	Microbial Numbers ($\log_e$)		Biofilm: Water Ratio	Z Statistic	Probability of Similarity (P = 0.05)
		Biofilm	Water			
Bruce	27	3.05	-0.92	53	6.60	<0.0001
B	61	2.92	-1.51	85	7.40	<0.0001
B	131	6.59	2.14	86	10.33	<0.0001
Nanticoke	27	4.88	0.68	67	7.95	<0.0001
N	61	7.17	3.23	51	6.57	<0.0001
N	131	8.86	5.34	34	8.26	<0.0001
Pickering	27	4.49	0.41	60	7.45	<0.0001
P	61	4.75	-0.05	122	8.00	<0.0001
P	131	7.48	4.04	31	7.98	<0.0001

TABLE 6

PSEUDOMONADS - APPROXIMATE TEST FOR DIFFERENCES BETWEEN LAKEWATER AND BIOFILM

Cooling Water Source	Time (Days)	Microbial Numbers ($\log_e$)		Biofilm: Water Ratio	Z Statistic	Probability of Similarity (P = 0.05)
		Biofilm	Water			
Bruce	27	10.09	7.77	10	5.32	<0.0001
B	61	10.93	8.58	11	5.39	<0.0001
B	131	15.43	12.73	15	6.01	<0.0001
Nanticoke	27	10.22	8.16	8	4.65	<0.0001
N	61	10.59	9.02	5	3.63	<0.0001
N	131	15.83	11.79	57	9.09	<0.0001
Pickering	27	10.25	7.79	12	5.73	<0.0001
P	61	12.65	8.56	60	9.15	<0.0001
P	131	16.92	12.81	61	9.31	<0.0001

APPENDIX D

MICROBIAL SPECIATION CALCULATIONS

TABLE 1

PROPORTION OF MICROBIAL GROUPS IN SOLID AND WATER PHASES IN THREE BIOREACTORS

Bioreactor Bruce B Nanticoke N Pickering P	Phase Biofilm BF Water W	Time (Days)	Percentage of Microbial Group					
			Aerobic Heterotrophs	Pseudomonads	Actinomycetes	Fungi	Ammonifiers	Sulphate Reducers
B	BF	27	3.49	8.55	81.38	0.01	5.98	0.60
B	BF	61	58.55	2.76	38.09	0.00	0.55	0.04
B	BF	131	45.41	25.99	27.92	0.00	0.59	0.09
B	W	0	29.64	48.73	12.21	0.02	9.16	0.24
B	W	27	14.84	12.32	66.65	0.00	5.99	0.20
B	W	61	45.75	2.58	51.11	0.00	0.44	0.12
B	W	131	47.91	29.62	21.70	0.00	0.61	0.15
N	BF	27	59.96	18.22	13.08	0.09	5.37	3.28
N	BF	61	93.78	2.58	2.73	0.08	0.72	0.10
N	BF	131	64.06	16.12	19.13	0.02	0.58	0.09
N	W	0	32.95	52.56	12.94	0.01	1.46	0.08
N	W	27	65.07	19.52	10.60	0.01	3.47	1.34
N	W	61	73.14	18.43	0.45	0.06	7.56	0.36
N	W	131	45.48	36.5	15.85	0.06	0.17	1.94
P	BF	27	39.07	22.73	20.66	0.07	13.52	3.96
P	BF	61	89.53	8.45	0.76	0.00	1.18	0.07
P	BF	131	29.35	31.39	25.55	0.00	13.68	0.03
P	W	0	51.56	23.55	7.99	0.21	15.17	1.52
P	W	27	28.11	16.39	6.12	0.01	47.74	1.63
P	W	61	93.86	4.45	0.51	0.00	0.97	0.21
P	W	131	45.15	18.38	35.14	0.00	1.21	0.12

TABLE 2

TEST FOR DIFFERENCES IN PROPORTION OF MICROBIAL GROUPS BETWEEN BIOFILM AND WATER PHASES

Bioreactor Bruce B Nanticoke N Pickering P	Time (Days)	Log_e Water Value-Log_e Biofilm Value						Pooled Variance	Corrected Sum of Squares	Chi Statistic	Probability of Exceeding Absolute χ Value
		Aerobic Heterotrophs	Pseudomonads	Actinomycetes	Fungi	Ammonifiers	Sulphate Reducers				
B	27	-1.24	-2.31	-2.88	-3.96	-2.68	-3.78	0.74	5.00	6.78	0.2372
B	61	-2.56	-2.36	-2.02	-4.44	-2.53	-1.33	0.67	5.37	7.99	0.1568
B	131	-2.76	-2.68	-3.07	-4.46	-2.79	-2.30	0.71	2.81	3.97	0.5530
N	27	-2.07	-2.04	-2.33	-4.19	-2.56	-3.02	0.80	3.31	4.16	0.5268
N	61	-3.77	-1.57	-5.34	-3.94	-1.20	-2.24	0.66	12.83	19.57	0.0015*
N	131	-5.20	-4.05	-5.05	-3.52	-6.07	-1.82	0.81	11.30	13.87	0.0165*
P	27	-2.46	-2.46	-3.35	-4.07	-0.87	-3.02	0.79	5.86	7.37	0.1948
P	61	-3.39	-4.11	-3.87	-4.80	-3.65	-2.42	0.78	3.15	4.02	0.5460
P	131	-3.15	-4.13	-3.28	-3.43	-6.00	-2.11	0.72	8.56	11.89	0.0363*